World Journal of

Gastroenterology

Contents

Contents

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Dar-In Tai, MD, PhD, Attending Doctor, Chief Doctor, Professor, Department of Gastroenterology and Hepatology, Chang Gung Memorial Hospital, Taipei 105, Taiwan

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 642 experts in gastroenterology and hepatology from 59 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

EDITORS FOR THIS ISSUE

REVIEW

Role of microRNAs in the main molecular pathways of hepatocellular carcinoma

Francesco Vasuri, Michela Visani, Giorgia Acquaviva, Thomas Brand, Michelangelo Fiorentino, Annalisa Pession, Giovanni Tallini, Antonia D'Errico, Dario de Biase

Francesco Vasuri, Michelangelo Fiorentino, Antonia D'Errico, Pathology Unit, Department of Experimental, Diagnostic and Specialty Medicine (DIMES), S.Orsola-Malpighi Hospital, University of Bologna, Bologna 40138, Italy

Michela Visani, Giorgia Acquaviva, Giovanni Tallini, Department of Medicine (Dipartimento di Medicina Specialistica, Diagnostica e Sperimentale), Molecular Diagnostic Unit, Azienda USL di Bologna, University of Bologna - School of Medicine, Bologna 40138, Italy

Thomas Brand, Department of Pharmacy and Biotechnology (Dipartimento di Farmacia e Biotecnologie), University of Bologna, Bologna 40127, Italy

Annalisa Pession, Dario de Biase, Department of Pharmacy and Biotechnology (Dipartimento di Farmacia e Biotecnologie), Molecular Diagnostic Unit, Azienda USL di Bologna, University of Bologna, Bologna 40138, Italy

ORCID number: Francesco Vasuri (0000-0002-1145-6025); Michela Visani (0000-0002-9051-2231); Giorgia Acquaviva (0000-0002 -8811-7865); Thomas Brand (0000-0001-5687-1399); Michelangelo Fiorentino (0000-0002-1749-150X); Annalisa Pession (0000-0001-9035-9698); Giovanni Tallini (0000-0003 -0113-6682); Antonia D'Errico (0000-0003-2747-3732); Dario de Biase (0000-0002-0609-8817).

Author contributions: Vasuri F, Visani M, Acquaviva G and de Biase D contributed to review conception and design, drafting of the manuscript, final approval of the manuscript; Fiorentino M, Pession A, Tallini G and D'Errico A contributed to review conception and design, critical revisions of important intellectual content of the manuscript, final approval of the manuscript; Brand T performs language editing and final approval of the manuscript.

Conflict-of-interest statement: The authors declare no financial or other conflicts of interest related to the submitted manuscript.

Abstract

Hepatocellular carcinoma (HCC) is the most common primary liver malignant neoplasia. HCC is characterized by a poor prognosis. The need to find new molecular markers for its diagnosis and prognosis has led to a progressive increase in the number of scientific studies on this topic. MicroRNAs (miRNAs) are small non-coding RNA that play a role in almost all main cellular pathways. miRNAs are involved in the regulation of expression of the major tumor-related genes in carcinogenesis, acting as oncogenes or tumor suppressor genes. The aim of this review was to identify papers published in 2017 investigating the role of miRNAs in HCC tumorigenesis. miRNAs were classified according to their role in the main molecular pathways involved in HCC tumorigenesis: (1) mTOR; (2) Wnt;

(3) JAK/STAT; (4) apoptosis; and (5) MAPK. The role of miRNAs in prognosis/response prediction was taken into consideration. Bearing in mind that the analysis of miRNAs in serum and other body fluids would be crucial for clinical management, the role of circulating miRNAs in HCC patients was also investigated. The most represented miRNA-regulated pathway in HCC is mTOR, but apoptosis, Wnt, JAK/STAT or MAPK pathways are also influenced by miRNA expression levels. These miRNAs could thus be used in clinical practice as diagnostic, prognostic or therapeutic targets for HCC treatment.

Key words: MicroRNA; Molecular pathway; mTOR; Prognosis; Hepatocellular carcinoma; Review

Core tip: Hepatocellular carcinoma (HCC) is the most common primary liver neoplasia and is characterized by a poor prognosis. MicroRNAs (miRNAs) are involved in the regulation of expression of the major tumor-related pathways in carcinogenesis and may act as oncogenes or tumor suppressor genes. mTOR is the most represented miRNA-regulated pathway in HCC. miRNAs found to be deregulated in HCC could be used in clinical practice as diagnostic, prognostic or therapeutic targets.

INTRODUCTION

Hepatocellular carcinoma (HCC), the most common primary liver malignant neoplasia, is a very diffuse malignancy, with variable incidence according to geography, and represents the fifth cause of cancer-related death worldwide[1,2]. Generally, HCC is characterized by a poor prognosis, mainly due to the limited treatment choices[3]: prognosis after surgery - including liver transplantation - depends on the tumor stage, the association with cirrhosis and on liver function[4]. Histology can be of little help in predicting the response to surgery, since the classic well-established histological features of HCC are seldom evaluable on liver needle biopsy: microvascular invasion (MVI) is rarely seen on biopsy, and tumor grade and architecture are too heterogeneous in HCC to be assessed on the basis of small sampling. Up to now, the most reliable prognostic markers after surgery are clinical and surgical, *e.g.*, the complete resection of the lesion, liver function tests,

and alpha-fetoprotein[5]. For liver transplantation, the inclusion within the Milan criteria remains a cornerstone for the good outcome of the recipients[6].

As for the systemic therapy for HCC, no serious options have been available until 2007, when the neoangiogenesis agent Sorafenib was introduced. Sorafenib gave one more chance to those patients with high-stage and MVI-positive HCC, not suitable for surgery[7,8]. Again, no predictive tests are available to assess which patients will benefit most from Sorafenib therapy.

The need to find new tissue and/or serum markers for HCC diagnosis and prognosis progressively increased the number of studies on the molecular mechanism behind liver carcinogenesis. The issue, however, still remains tangled, due to the high heterogeneity of HCC (not only in phenotype), and to the complex multistep carcinogenesis occurring differently in cirrhotic and non-cirrhotic livers[2].

MicroRNAs (miRNAs) are small non-coding RNA (20-25 nucleotides) that play a role in almost all main cellular pathways[9]. miRNAs contribute to a variety of physiological and pathological events, including several types of tumors[10-15]. Thus, miRNAs are involved in the regulation of expression of the major tumor-related genes in carcinogenesis, acting as oncogenes or tumor suppressor genes[16].

The aim of this review was to identify papers published in the last 12 mo (from January 2017 to December 2017), investigating the possible role of miRNAs in HCC tumorigenesis. miRNAs were classified according to their role in the main molecular pathways involved in HCC: (1) mTOR; (2) Wnt; (3) JAK/STAT; (4) apoptosis; and (5) MAPK. Moreover, the possible role of miRNAs in prognosis/response prediction and the level of circulating miRNAs in HCC patients were also investigated.

MIRNAS IN HCC MOLECULAR PATHWAYS

mTOR pathway

mTOR (mammalian target of rapamycin) is a well conserved serine-threonine kinase that plays a fundamental role in the signaling network that controls growth and cell metabolism. mTOR is the physical target of rapamycin. mTOR exists in two different multi-protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is composed of five components: mTOR, Raptor, PRAS40, GβL, and DEPTOR[17]. mTORC1 is directly inhibited by rapamycin but, at the same time, it is modulated by genotoxic stress, growth factors, oxygen and energy status, resulting in modulation of cell growth and proliferation[18]. These inputs indirectly regulate mTORC1 by controlling the activation status of TSC1-TSC2. For example, growth factors block TSC1-TSC2 and mTORC1 is consequently activated. On the contrary, energy deficit, genotoxic stress or oxygen

deprivation are positive signals on TSC1-TSC2, which inhibit mTORC1. Activated mTORC1 promotes protein synthesis and lipid biogenesis, it controls mitochondrial metabolism and biogenesis; it also inhibits catabolism by blocking autophagy and it inhibits growth factor signaling by activating negative feedback loops that block the PI3K pathway[18-20].

The second complex, mTORC2, is composed of six components: mTOR, Rictor, GβL, Sin1, PRR5/Protor-1, and DEPTOR. mTORC2 regulates cellular survival, cytoskeletal organization and metabolism. Compared to mTORC1, the mechanisms of mTORC2 are less understood: It is insensitive to acute treatment with rapamycin, but it was reported that a long-term treatment with rapamycin reduces mTORC2 signaling, suppressing the assembly of the complex[19,21]. This complex is activated by growth factors with a PI3K-dependent mechanism and the work of Zinzalla *et al*[22] suggested a possible role of ribosomes for mTORC2 complex activation[20,22].

Due to its key role in regulating cell growth, survival and metabolism, the mTOR pathway is aberrantly activated in many diseases, including cancer, cardio-vascular disease and diabetes[20]. In up to 50% of HCC cases, an aberrant activation of mTOR was reported, mainly downstream of the insulin growth factor (IGF) or epidermal growth factor (EGF) signaling cascades[23].

Many studies showed tumor suppressor miRNAs, the down-regulation of which lead to the mTOR pathway activation in HCC cells.

miR-758-3p: Jiang *et al*[24] showed that the restoration of miR-758-3p in HCC cell line could suppress cell proliferation, migration, and invasion. miR-758-3p markedly down-regulates the expression of MDM2 and mTOR and, at the same time, the expression of p53, AKT and PRAS40 resulted up-regulated. mTOR can be regulated by its upstream effector AKT, which can suppress PRAS40 so as to eliminate the inhibition it exerts on mTORC1[24].

miR-142: Yu *et al*[25] demonstrated that miR-142 expression was reduced in 50 tumor tissues in comparison to correspondent normal tissues and in two HCC cell lines compared to human normal liver cell line. This lower expression was linked to poor clinical parameters like high TNM stage and distant metastasis. miR-142 was identified to directly target the transforming growth factor β (TGF-β), which controls cell vitality, proliferation, epithelial-mesenchymal transition (EMT) and neo-angiogenesis. mTOR is one of the effector pathways of TGF-β signaling. These findings imply that miR-142 is a tumour suppressor gene in HCC and that it increases the TGF-β-induced development of hepatocellular carcinoma[25].

miR-199b-5p: In 100 pairs of HCC patients' tumor tissues and adjacent liver tissues a significant down-regulation of miR-199b-5p was observed and associated to poor clinical outcome. N-cadherin was the demon-strated target of miR-199b-5p and it promoted EMT in HCC cells. The restoration of miR-199b-5p suppressed cell migration, invasion and metastasis in xenograft tumors. It was demonstrated that the miR-199b-5p overexpression lead to suppression of TGF-β1-induced Akt phosphorylation. Moreover, inhibition of the PI3K/Akt signaling pathway blocked TGF-β1-induced N-cadherin overexpression in HCC cells. The inhibitory effects on EMT and on the TGF-β1 signaling pathway support the potential use of miR-199b-5p as a promising strategy to treat HCC[26].

miR-187, miR-497, miR-99a, miR-592: IGF-1R activation, through the PI3K/Akt/mTOR axis, is responsible for cell proliferation, migration and invasion in HCC[27]. IGF-1R is a target of miR-187, miR-497, miR-99a and miR-592[28-30]. miR-187 was found downregulated in HCC tissues and cell lines: as reported by Han *et al*[29], the restoration of miR-187 leads to a significant arrest of HCC growth. miR-497 and miR-99a target the 3′-UTR of both IGF-1R and mTOR and were shown to be down-regulated in HCC human tissues and cell lines: the co-transfection with both miRNAs slowed cell proliferation and the tumor growth in HCC cell lines and in xenograft models[28]. Wang and colleagues demonstrated that miR-592 was significantly downregulated in HCC tissues and cell lines and that its low expression was associated with lymph node metastases[30]. These results indicated that miR-187, miR-497, miR-99a, miR-592 could be investigated as potential therapeutic targets for HCC in the future.

miR-296-5p: The low expression of miR-296-5p is directly linked to the activation of the mTOR pathway in HCC growth. Gain-of-function experiments demonstrated that miR-296-5p inhibited HCC cell proliferation, migration and invasion *in vitro*, by targeting AKT2. These findings indicated that the miR-296-5p/AKT2 axis plays important roles in HCC carcinogenesis and progression, and that miR-296-5p/AKT2 could be considered a potential target for HCC therapy[31].

miR-139-5p: PDK1/AKT/mTOR axis activation could lead to hepatocellular carcinoma cell proliferation. PDK1 is a known target of miR-139-5p, found down-regulated in HCC tissues and cell lines. Mo *et al*[32] also observed that miR139-5p/PDK1 expression was regulated by long-coding RNA XIST, which was found over-expressed in HCC.

miR-15b-5p: Opa interacting protein 5 (OIP5) was found up-regulated in HCC, inducing tumor growth and metastasis *in vitro* and *in vivo*. OIP5 induces mTORC1 and GSK-3β/β-catenin signaling activation, through AKT. miR-15b-5p was found down-regulated in HCC cells and OIP5 was found to be its direct target. These findings suggest that the restoration of miR-15b-5p could inhibit

OIP5-mediated oncogenic signaling in HCC[33].

miR-345: Yu and colleagues found that the expression of miR-345 was significantly down-regulated in 65 HCC cases, and matching tumor-adjacent tissues, and in HCC cell lines. They also reported a clinical correlation between the low expression of miR-345 and venous infiltration, multiple lymph node metastases, and advanced TNM stage. The restoration of miR-345 inhibited migration and invasion ability of HCC cells. It was demonstrated that interferon regulatory factor 1 (IRF1) was a direct target of miR-345 and that IRF-1 mediated the oncogenic effects triggering mTOR/STAT3/AKT signaling[34].

miR-223: Dong and colleagues showed that miR-223 was able to suppress cell growth and to promote apoptosis in HCC cell lines (HepG2 and Bel-7402). Ras-related protein Rab-1(Rab1) is specifically regulated by miR-223. These data suggested that, in HCC cells, the anti-tumor effects due to miR-223 restoration may be due to the inactivation of the mTOR pathway, caused by the suppression of Rab1 when miR-223 is over-expressed. According to these results, miR-223 may be a potential therapeutic target for treating HCC, mediating mTOR signaling silencing[35].

Other studies showed oncogenic miRNAs, the upregulation of which leads to the mTOR pathway activation in HCC cells.

miR-33a: The levels of miR-33a were observed as significantly higher in HCC tissues than in adjacent non-tumor tissues. This elevated expression of miR-33a correlated with adverse clinical features and poor prognosis. It was demonstrated that miR-33a could promote cell growth by modulating the proliferation and apoptosis of HCC cells. Its direct target is PPARα, one of the targets of mTORC1[36].

miR-302d: Chen and colleagues demonstrated that the overexpression of miR-302d promoted cell growth and migration and suppressed apoptosis in HCC cell lines and that it promotes xenograft tumor growth *in vivo*. These mechanisms were found to be mediated by TGFbR2-signaling, a target of miR-302d[37].

miR-23b: miR-23b was found to be significantly up-regulated in tumor tissues of HCC patients. It was demonstrated that this miRNA regulated ST7L, a suppressor of the AKT/GSK3β/β-catenin pathway in HCC cells. MiR-23b thus acts as an oncomir in HCC, stimulating proliferation and metastasis through the mTOR and β-catenin signaling cascades[38].

miR-181a, miR-155-5p, miR-25: miR-181a was found to be up-regulated in HCC tissues compared to adjacent tissues; moreover, its levels were dramatically higher in metastatic HCC tissues than in non-metastatic HCC tissues. miR-181a regulates the proliferation and

invasion of HCC cells by targeting PTEN, the reduction of which activates the PI3K/Akt pathway[39]. Another miRNA was found to be over-expressed in HCC and plays an oncogenic role in HCC by targeting PTEN: miR-155-5p promotes cell growth, migration and invasion, but inhibits apoptosis in vitro and promoted HCC progression in vivo[40]. PTEN is a known target also of miR-25, which is over-expressed in HCC cell lines and in liver cancer stem cells (LCSCs)[41].

Wnt signaling pathway

β-catenin phosphorylation and degradation and its regulation by Wnt are the essence of the Wnt pathway. Signaling by the Wnt family proteins is one of the fundamental mechanisms that induce cell proliferation, cell polarity and cell fate during development and tissue homeostasis (Logan and Nusse, 2004). Canonical Wnt signaling functions by regulating the amount of β-catenin. In the absence of Wnt, cytoplasmic β-catenin protein is degraded by the Axin complex. This non-stop degradation prevents β-catenin from reaching the nucleus, and Wnt target genes are thereby repressed[42-44]. Inhibitors of Wnt signaling might be effective in HCC, where mutations in the Wnt pathway components are quite common. In the HepG2 cell line, knockdown of β-catenin, mediated by RNA interference, decreased proliferation and growth *in vitro*[45,46] (Figure 1).

miRNA-10a, miR-30e, miR-215, miR-125b and miR-148a: Ashmawy and colleagues observed that 11 miRNAs (miR-10a, miR-106b, miR-99a, miR-148a, miR-125b, miR-30e, miR-199a, miR-199a3p, miR-24, miR-122 and miR-215) were down-regulated in HCC patients. Five of these miRNAs (miRNA-10a, miR-30e, miR-215, miR-125b and miR-148a) were also associated with the expression of genes involved in the Wnt/β-catenin pathway, such as β-catenin, APC and c-myc[47].

miR-155 and miR-183: In the same study, the authors detected that miR-155 and miR-183 were up-regulated in HCC patients if compared to controls and that miR-155 was correlated with liver cirrhosis[47].

miR-18a: Other than miR-155 and miR-183, other miRNAs involved in the Wnt/b-catenin pathway were observed up-regulated in HCC. Liu *et al*[48] identified that miR-18a expression was upregulated in human HCC if compared to the adjacent non-tumoral liver tissue. This up-regulation promotes the proliferation and migration of HCC cell lines by inhibiting KLF4, a factor that negatively regulates β-catenin expression. This data led the authors to hypothesize that miR-18 could be a therapeutic target for HCC treatment[48].

miR-195: Yan and colleagues showed a potential application of miR-195 in the cancer therapy of HCC. They observed that miR-195 was markedly down-

Figure 1 Wnt signaling. MicroRNAs deregulated in hepatocellular carcinoma and involved in Wnt signaling pathway.

regulated both in HCC cell lines and in 36 HCCs compared to adjacent non-tumoral liver tissues. The expression of miR-195 was inversely correlated with AEG-1 expression, which was demonstrated to be a target of miR-195. Overexpression of AEG-1 activates the PI3K/Akt, nuclear factor-κB, and Wnt/β-catenin signaling pathways stimulating proliferation, metastasis, angiogenesis and chemoresistance[49]. An analysis performed using miR-195 mimics showed how miR-195 inhibited liver cancer cell growth and induced apoptosis in HCC cell lines. The overexpression of miR-195 also decreased tumor growth of hepatoma xenografts in nude mice[50].

miR-320a: miR-320a was observed to be down-regulated in HCC tissues if compared to paired adjacent non-tumoral liver tissues. In samples with miR-320a inhibition, an up-regulation of the expression levels of β-catenin, c-myc, cyclin D1 and DKK-1 was observed. According to this data miR-320a may be considered as a tumor-suppressive microRNA in human HCC, through the down-regulation of the β-catenin pathway[51]. miR320a was observed to be down-regulated also in a cohort of 50 HCC tissues by Xie and colleagues. The authors also identified c-Myc as a direct target of miR-320a and observed that inducing upregulation of miR-

320a in HCC leads to inhibition of HCC cell proliferation and invasion capability through c-Myc silencing. These data support the role of miR320a as a tumor-suppressive microRNA in HCC and provide evidence that miR-320a may be used as a potential target for HCC treatment[52].

miR-98-5p: Another miRNA observed to be down-regulated in HCC tissues is miR-98-5p. Down-regulation of miR-98-5p correlates with tumor size, lymph node metastasis, and clinical stage. In addition, HCC patients with low expression of miR-98-5p had a shorter survival time compared to those with high miR-98-5p levels. miR-98-5p down-regulation in HCC has been associated with up-regulation of Insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) in HCC, which induces cell proliferation while inhibiting cell apoptosis[53].

miR-15b-5p: As reported above (see mTOR pathway paragraph), miR-15b-5p is involved in AKT/mTOR pathway. However, its expression also inhibits GSK-3β/β-catenin signaling in HCC[33].

miR-1247-5p: miR-1247-5p levels are down-regulated in patients with HCC and in HCC cell lines. This downregulation is probably due to hypermethylation

of miR-1247-5p gene. Also, the overexpression of miR-1247-5p inhibits the invasion and proliferation of HepG2 cells, induces cell apoptosis *in vitro*, and suppresses the growth of transplanted tumors *in vivo*. Wnt3 is a target of miR-1247-5p and overexpression of miR-1247-5p significantly down-regulated its expression. The evidence that the expression of miR-1247-5p can be regulated by methylation indicates that miR-1247-5p may be a potential therapeutic target for HCC[54].

JAK-STAT pathway

The JAK/STAT pathway regulates development and it is involved in stem cell maintenance, hematopoiesis and inflammatory response. Active JAKs recruit STAT (signal transducers and activators of transcription) proteins that form dimers that translocate to the nucleus when phosphorylated. These dimers modulate transcription of genes involved in differentiation, proliferation and apoptosis. The JAK/STAT pathway is regulated at multiple levels. Many cancers and neoplastic cells employ several strategies to activate the JAK/STAT pathway (*e.g.*, activating mutations in STATs or reduced expression of negative regulators[55-57]) (Figure 2).

miR-214-3p: miR-214-3p is expressed at low levels in HCC[58]. PIM-1 is an oncogene encoding for a serine/threonine kinase protein involved in several human cancers. PIM-1 transcription is initiated by STAT proteins and plays a key role in signal transduction, contributing to both cell proliferation and survival, and thus providing an advantage in tumorigenesis[59]. PIM-1 is a miR-214-3p target and PIM-1 expression is enhanced in HCC[58].

miR-30e: miR-30e is down-regulated in the majority of HCC tissues. Restoration of its expression down-regulates JAK1 expression levels. Silencing JAK1 inhibits migration, proliferation and invasion of HCC cells. For this reason, miR-30e might be a prognostic marker of HCC and a putative therapeutic target[60].

miR-340: JAK-1 was also identified as a direct target of miR-340. miR-340 was found to be significantly down-regulated in HCC tissues and cell lines and, *in vitro,* its overexpression inhibited migration, cell proliferation and invasion[61].

miR-140-5p and miR-200: miR-140-5p and miR-200 were down-regulated in HCC and predicted to target Pin1[62]. Pin-1 is an independent factor for poor prognosis in HCC, it is overexpressed in ~70% of human HCCs[63], and it is correlated with larger tumor size, higher incidence of MVI and poor prognosis in HCC[64]. The over-expression of miR-140-5p inhibits human HCC cell growth, colony formation and migration[62].

miR-638: miR-638 expression in HCC tissues is down-regulated if compared to the paired non-tumoral tissues. Low expression of miR-638 was linked to venous infiltration and TNM stage. Moreover, low levels of miR-638 are associated with a lower E-cadherin and vimentin expression if compared to cells showing high miR-638 levels. miR-638 down-regulation increases SOX2 expression, a gene overexpressed in HCC and involved in oncogenesis and in the progression of various cancers[65,66]. Moreover, SOX2 expression is associated with overall poor survival in HCC patients and it promotes cancer cell invasion[67]. These data lead to considering miR-638-SOX2 as a putative target for repressing the development and metastasis of HCC[68].

Apoptosis

Apoptosis occurs normally during development to maintain cell populations in tissues and as a defense mechanism in immune reactions or when cells are damaged[69,70]. TP53 was the first tumor suppressor gene to be linked to apoptosis and it is well established that TP53 mutations occur in the vast majority of human cancers[71]. In fact, if on one hand wild type p53 promotes apoptosis, cell-cycle arrest and senescence, the loss of p53 function increases viability, chromosomal instability and cellular lifespan. Disruption of the apoptotic pathway correlates with the progression of several tumors[72].

miR-30a: Anoikis is a form of cell death due to loss of contact of the cells with the extracellular matrix. Low levels of miR-30a were observed in several HCC cell lines (Hep3B, HepG2, SMMC-7721, MHCC97-L, MHCC97-H and HCCLM3) and in HCC tumor tissue compared to adjacent non-neoplastic tissue. miR-30a silencing is accompanied by an increase in the expression of Beclin 1 and Atg5 proteins and by a decreased number of cells undergoing anoikis[73].

miR-365: miR-365 expression was significantly lower in HCC cells (SMC7721, HepG2, Bel7404 and Bel7402) compared to a normal hepatocellular cell line (LO2). Inducing up-regulation of miR-365 leads to a significant decrease in cellular activity and to the inhibition of tumor growth. It has also been observed that in cells with an up-regulation of miR-365, the expression of Bax, cyto C and cleaved caspase 3, which is downstream of Bcl-2, were also markedly up-regulated[74].

miR-526a: miR-526a is down-regulated in HCC tissues. In vitro, the introduction of miR-526a into HCC cell lines significantly decreased HCC proliferation, migration and invasion, via p21 inactivation[75].

miR-377: miR-377 was down-regulated in HCC tumors if compared to the adjacent non-neoplastic tissue. *In vitro* experiments with HCC cell lines demonstrated that a gain of miR-377 function inhibited colony formation, suggesting that miR-377 plays a key role as a tumor suppressor in HCC. In cells with high levels of miR-377 the apoptotic rate was significantly higher than in the

Figure 2 JAK/STAT pathway. MicroRNAs deregulated in hepatocellular carcinoma and involved in JSK/STAT pathway.

controls. Bcl-xL is an anti-apoptotic protein and it is overexpressed in about 33% of HCC[76], conferring resistance to apoptosis. The higher level of apoptotic rate observed in cell lines with miR-377 over-expression was associated with a concomitant down-regulation of Bcl-xL mRNA levels, suggesting that Bcl-xL is a target of miR-377[77].

miR-199a-5p: miR-199a-5p was down-regulated in HCC tissues compared to pair-matched non-neoplastic hepatic tissues[78], and the same was the case for let-7c expression[79]. miR-199a-5p down-regulation was correlated with tumor size and invasion. Moreover, the low expression of miR-199a-5p and let-7c was associated with higher metastatic capability in HCC cell lines. MAP4K3 is a pro-apoptotic kinase that activates the Intrinsic Apoptosis Pathway[80]. MAP4K3 gene was predicted as a possible target of miR-199a-5p and let-7c and the up-regulation of both miRNAs leads to a significant decrease in MAP4K3 protein level, resulting also in a decrease in HCC cell migration and invasion[78].

miR-330: miR-330 level was higher in HCC tissues if compared to adjacent non-neoplastic specimens. The up-regulation of miR-330 was associated with shorter survival in HCC patients. ING genes have been reported to be implicated in apoptosis, cell cycle regulation,

and DNA repair. ING4 plays important roles in many cancer-related processes, such as apoptosis, cell proliferation and growth, angiogenesis and migration. ING4 expression is decreased in several cancers[81]. Overexpression of miR-330 reduced the expression of ING4 in HCC cells promoting HCC cell proliferation and invasion[82].

MAPK cascade
The mitogen-activated protein kinase (MAPK) pathway is characterized by different kinase proteins that link extracellular signals to the machinery that controls physiological cellular processes such as growth, differentiation, proliferation, migration and apoptosis. Alterations in the MAPK cascade impinge on almost all previously listed physiological processes and play a critical role in the development and progression of cancer[83] (Figure 3).

miR-346: miR-346 was down-regulated in HCC tissues and its expression levels are associated with tumor size and TNM stage. An *in vitro* study revealed that the loss of miR-346 leads to the up-regulation of S phase in HCC cell lines. One of the putative targets of miR-346 is SMYD3. SMYD3 is an oncogene up-regulated in several tumors, including HCC[84]. SMYD3 expression leads to methylation of MAP3K2 by increasing MAP kinase

Figure 3 MAPK cascade. MicroRNAs deregulated in hepatocellular carcinoma and involved in MAPK cascade.

signaling and promoting the formation of Ras-driven carcinomas[85]. Restoring miR-346 levels in HCC cell lines prevented proliferation through the suppression of SMYD3 expression[86].

miR-143: miR-143 expression was reduced in HCC tumor tissues and human liver cancer cell lines (SMMC-7721, Hep3B, HepG2, Huh7, Bel7402, MHCC97-H and SK-Hep1) compared to non-neoplastic adjacent tissues and normal liver cell line (L02), respectively[87]. GATA-binding factor 6 (GATA6) is a transcriptional factor with an oncogenic role in various types of tumor, favoring cancer progression[88]. GATA6 is a potential target for miR-143 and its expression is downregulated by miR-143 overexpression in HepG2 and Bel7402 cells[87].

miR-125a: miR-125a was detected as down-regulated in 80% of HCC biopsies if compared with the adjacent non-tumor liver tissue. When grouping patients according to HCC etiology, miR-125a was downregulated in 80% of HBV patients, 78% of HCV patients, and in all 4 patients with non-alcoholic steatohepatitis. *In vitro* analysis revealed that MMP11, SIRT7 and c-Raf were the main

miR-125a targets. In support of this, MMP11, SIRT7 and c-Raf were up-regulated in about 80% patients with miR-125a down-regulation, hinting at an oncosuppressor effect of the microRNA through the regulation of MMP11, c-Raf and SIRT7 expression[89].

miR-217: miR-217 expression levels in HCC tissues were significantly decreased, while MTDH levels were significantly upregulated. miR-217 up-regulation in HCC cell lines lead to a remarkable downregulation of MTDH mRNA expression, demonstrating that MTDH is a target of miR-217. Metadherin (MTDH) is a transmembrane protein overexpressed in several cancers[90] and it is associated with tumor development and with aggressive course[49]. In cell lines with miR-217 up-regulation and MTDH down-regulation, cell apoptosis notably increased, indicating that miRNA expression promotes apoptosis of HCC cells[91].

miR-200a: miR-200a was down-regulated in HCC cell lines and tissues and it was correlated with the metastatic ability of HCC[92]. GAB1 is one of the molecules targeted by miR-200a. The overexpression of miR-200a suppressed the levels of GAB1 in HCC cell

Table 1 List of the microRNAs involved in at least one of the main molecular pathways, and with a clinical significance, according to the present review

miRNAs (regulation)	Pathway	Clinical
99a	mTOR	Recurrence after resection
497		Recurrence after resection, recurrence after transplantation
195		Recurrence after resection
140-5p		Recurrence after resection
23b		Recurrence after resection
223		Recurrence after resection
199a-5p		Recurrence after transplantation
181a-5p		Resistance to Sorafenib/CHT
33a-5p		Resistance to Sorafenib/CHT
125b	Wnt	Recurrence after resection, recurrence after transplantation
195		Recurrence after resection
18 (up)		Recurrence after transplantation
140-5p	JAK-STAT	Recurrence after resection
365	Apoptosis	Recurrence after resection
125a	MAPK	Recurrence after resection, resistance to Sorafenib/CHT

lines, inhibiting cell migration and invasion[92].

TISSUE miRNA AND POST-SURGICAL OUTCOME

Liver resection
In a wide-array miRNA analysis on resected patients with low-stage HCC (within the Milan criteria), Sato *et al*[93] found that the deregulation of 13 intratumoral miRNAs (miR-100, miR-99a, miR-99b, miR-125b, miR-378, miR-129-5p, , miR-125a-5p, miR-497, miR-22, miR-140-3p, miR-145, miR-221, miR-195) significantly correlated with post-surgical (Table 1). The same is applies to the deregulation of more than 50 miRNAs in the non-tumoral tissue, among which the most significant was miR-96. miR-125b downregulation, in combination with more typical prognostic factors, was also correlated with low disease-free survival (DFS) after resection in later works[94] (Table 1).

In a more recent study on resected HCC, Lin *et al*[3] identified 16 miRNAs related to MVI, the hierarchical clustering of which was able to predict survival after resection: miR-452-5p, miR-378, miR-9-5p, miR-550a-5p, miR-15a-5p, miR-140-5p, let7g, miR-152-3p, miR-122-5p, miR-212-3p, miR-23b, miR-365a, miR-629-5p, miR-1270, miR-659-3p and miR-3941 (Table 1).

Other miRNAs whose down-regulation was correlated with poor prognosis after resection were miR342-3p[95], miR-655-3p[96], miR-105-1 *via* NCOA1 deregulation[97], miR-223 associated with an increased Stathmin-1 expression[98], and miR-483-3p in "histologically advanced" HCC (high-grade and/or MVI)[99]. Upregulation of miR-19b was correlated with good prognosis after resection in resected patients with advanced HCC[100]. Albeit miRNA down-regulation is often associated to worse prognosis, up-regulation is not always a good prognostic sign: for example, the up-regulation of miR-135a, miR-29a5p and miR-221 was significantly associated with early HCC recurrence[101-103] (Table 1).

Liver transplantation
The search for molecular predictors for HCC recurrence after orthotopic liver transplantation (OLT) is even more tangled than after resection, due to the particular immune status of the recipients and the intrinsic capability of HCC tumor cells not only to give metastases to distant organs, but also to implant in the graft. A wide microarray profiling by Barry *et al*[104] found more than 60 miRNAs to be deregulated in recurrent HCC after OLT. Interestingly, miR-125b and miR-497 - already mentioned above in post-resection recurrence - are among the most significant[93,104]. Common mechanisms beyond HCC recurrence after both resection and liver transplantation are likely to indicate that a more aggressive tumor biology leads to a higher risk of recurrence or dissemination.

Morita *et al*[105] demonstrated that the concomitant upregulation of miR-18a and down-regulation of miR-199a-5p correlated with the worst disease-free survival in a population of 70 transplanted patients. The most represented sites of recurrence were lymph nodes, lung and bone. The mechanisms proposed by the authors included the link between miR-18a and TNFα, as well as the regulation of the HIF1α, the VEGF-A, and the IGF pathways by miR-199a-5p[105] (Table 1).

Response to chemotherapy
The role of miRNAs in the response to chemotherapy is still largely unresolved, especially for HCCs, which are malignancies with an extreme molecular heterogeneity. Due to this heterogeneity, no target therapies specific for HCC have been available since the introduction of Sorafenib in 2005. Sorafenib changed the natural history of patients with advanced HCC not suitable for surgery[7], but no molecular markers for the prediction of the response to this therapy are available yet. Only few *in vitro* studies focusing on the role of miRNAs in the response to Sorafenib exist, *e.g.*, miR-137[106] and miR-125a-5p[107]. A very interesting recent study

found that the deregulation of miR-181a-5p in patients' serum was correlated with a worst disease control after Sorafenib therapy[108] (Table 1).

As for the resistance to classic chemotherapic drugs, other *in vitro* studies showed that miR-205-5p and miR-503 were involved in the resistance to 5-Fuorouracil[109,110], miR-33a-5p was involved in the resistance to Cisplatin[111], and miR-31 was involved in the resistance to Adriamycin[112]. An exhaustive *in vitro* and *in vivo* study by Jin *et al*[113] showed that the tissue levels of miR-26a/b regulated the mechanism of autophagy, thus influencing the cell's resistance to drugs. So, in spite of the many contributions in the literature about miRNAs and HCC, there is a lack of *in vivo* studies on the issue of the response to systemic therapy.

SERUM MIRNAS

The analysis of miRNAs in serum and other body fluids (*i.e.*, urines) would be crucial for clinical management, since it would allow diagnosis and/or prognosis of HCC patients before surgery. However, the study of serum miRNAs is still a complicated issue, due to the high tumor/patient variability and the lack of a standard control among laboratories. miR-122 - the miRNA most represented quantitatively in the human liver - is the most promising in early HCC diagnosis, albeit all authors generally agree that miR-122 serum levels also increase in non-neoplastic liver diseases[114]. The first proposed serum panel for HCC detection was miR-122 associated with miR-21 and miR-223[115]. A recent meta-analysis by Ding *et al*[116] showed that difficulties exist also in the comparison of scientific results among centers: the "high-frequency expression miRNAs" best suited for HCC diagnosis from serum were miR-122, miR-21, and miR-199, and generally a panel of multiple serum miRNAs is advisable. For example, a panel composed by three serum miRNAs (miR-92-3p, miR-3126-5p and miR-107) together with serum alpha-fetoprotein (AFP) showed higher sensitivity and specificity in the early diagnosis of HCC compared to AFP alone[117]. Another study found that serum miR-939, miR-595, miR-519d, and miR-494 were able to differentiate cirrhotic patients with and without HCC better than AFP[118]. Other serum miRNAs are likely to be useful in the diagnosis of local or distant HCC recurrence after surgery, like miR-486-5p[119] and miR-34a[120].

CONCLUSION

The extreme heterogeneity of HCC, in both its morphological picture (as assessed by radiologists and pathologists) and in its clinical course and outcome, reflects the heterogeneity of its bio-molecular status. Several molecular pathways are involved in hepatocarcinogenesis, as well as in the regenerative-dysplastic-neoplastic progression observed in cirrhotic nodules. As a consequence, the up- or down-regulation of several miRNAs is involved. As evidenced by the present review, the most represented miRNA-regulated pathway in HCC is mTOR, but other pathways, such as as apoptosis, Wnt or MAPK, are also influenced by miRNA expression levels. Moreover, as shown in Table 1, some miRNAs involved in at least one of the main molecular pathways of hepatocarcinogenesis are likely to have a clinical significance (recurrence after surgery, response to systemic therapy).

The identification of specific tissue and serum miRNAs, able to predict the arising of HCC in cirrhosis, to predict HCC recurrence after surgery, or to predict the response to systemic therapy, might lead to a drastic improvement in the management of these patients. Anyhow, the clinical application of miRNAs has always been complicated, especially because of inter-laboratory variability, due to the choice of control to be used for normalization. A recent study of our group showed how the miRNA profile of HCCs changed using a pool of cirrhotic tissues or a pool of healthy livers as non-tumor controls[121]. Other authors suggested to employ stable miRNAs as controls for the study of the expression of other miRNAs[122]. In the light of the available data, it would be useful to elaborate, based on the most representative miRNA in *in vivo* model (*e.g.*, rat, mouse), to better understand the possible role of these molecules as theragnostic markers in HCC. The issue is still open, and the standardization of miRNA analysis among laboratories is crucial for the development of a miRNA-based diagnosis of liver nodules, as well as of a miRNA-regulatory therapy.

REFERENCES

1 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin* 2016; **66**: 7-30 [PMID: 26742998 DOI: 10.3322/caac.21332]

2 **Loda M**, Mucci LA, Mittelstadt ML, Hemelrijck MV, Cotter MB, Springerlink (Online Service). Pathology and Epidemiology of Cancer. XIV 2017

3 **Lin Z**, Cai YJ, Chen RC, Chen BC, Zhao L, Xu SH, Wang XD, Song M, Wu JM, Wang YQ, Zhou MT, Shi KQ. A microRNA expression profile for vascular invasion can predict overall survival in hepatocellular carcinoma. *Clin Chim Acta* 2017; **469**: 171-179 [PMID: 28365450 DOI: 10.1016/j.cca.2017.03.026]

4 **Pang TC**, Lam VW. Surgical management of hepatocellular carcinoma. *World J Hepatol* 2015; **7**: 245-252 [PMID: 25729479 DOI: 10.4254/wjh.v7.i2.245]

5 **Bosman FT**, World Health Organization, International Agency for Research on Cancer. WHO classification of tumours of the digestive system. (4th). IARC, Lyon. 2010

6 **Mazzaferro V**, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, Ammatuna M, Morabito A, Gennari L. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 1996; **334**: 693-699 [PMID: 8594428 DOI: 10.1056/NEJM199603143341104]

7 **Bruix J**, Sherman M; American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53**: 1020-1022 [PMID: 21374666 DOI: 10.1002/hep.24199]

8 **Kudo M**. Systemic Therapy for Hepatocellular Carcinoma: 2017 Update. *Oncology* 2017; **93** Suppl 1: 135-146 [PMID: 29258077 DOI: 10.1159/000481244]

9 **Ambros V**. The functions of animal microRNAs. *Nature* 2004; **431**: 350-355 [PMID: 15372042 DOI: 10.1038/nature02871]

10 **de la Chapelle A**, Jazdzewski K. MicroRNAs in thyroid cancer.

J Clin Endocrinol Metab 2011; **96**: 3326-3336 [PMID: 21865360 DOI: 10.1210/jc.2011-1004]

11   **Wang W**, Luo YP. MicroRNAs in breast cancer: oncogene and tumor suppressors with clinical potential. *J Zhejiang Univ Sci B* 2015; **16**: 18-31 [PMID: 25559952 DOI: 10.1631/jzus.B1400184]

12   **Yonemori K**, Kurahara H, Maemura K, Natsugoe S. MicroRNA in pancreatic cancer. *J Hum Genet* 2017; **62**: 33-40 [PMID: 27251005 DOI: 10.1038/jhg.2016.59]

13   **Inamura K**, Ishikawa Y. MicroRNA In Lung Cancer: Novel Biomarkers and Potential Tools for Treatment. *J Clin Med* 2016; **5**: [PMID: 27005669 DOI: 10.3390/jcm5030036]

14   **Ishiguro H**, Kimura M, Takeyama H. Role of microRNAs in gastric cancer. *World J Gastroenterol* 2014; **20**: 5694-5699 [PMID: 24914330 DOI: 10.3748/wjg.v20.i19.5694]

15   **Visani M**, de Biase D, Marucci G, Cerasoli S, Nigrisoli E, Bacchi Reggiani ML, Albani F, Baruzzi A, Pession A; PERNO study group. Expression of 19 microRNAs in glioblastoma and comparison with other brain neoplasia of grades I-III. *Mol Oncol* 2014; **8**: 417-430 [PMID: 24412053 DOI: 10.1016/j.molonc.2013.12.010]

16   **Nana-Sinkam SP**, Croce CM. Clinical applications for microRNAs in cancer. *Clin Pharmacol Ther* 2013; **93**: 98-104 [PMID: 23212103 DOI: 10.1038/clpt.2012.192]

17   **Peterson TR**, Laplante M, Thoreen CC, Sancak Y, Kang SA, Kuehl WM, Gray NS, Sabatini DM. DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. *Cell* 2009; **137**: 873-886 [PMID: 19446321 DOI: 10.1016/j.cell.2009.03.046]

18   **Laplante M**, Sabatini DM. mTOR signaling at a glance. *J Cell Sci* 2009; **122**: 3589-3594 [PMID: 19812304 DOI: 10.1242/jcs.051011]

19   **Laplante M**, Sabatini DM. mTOR signaling in growth control and disease. *Cell* 2012; **149**: 274-293 [PMID: 22500797 DOI: 10.1016/j.cell.2012.03.017]

20   **Zoncu R**, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* 2011; **12**: 21-35 [PMID: 21157483 DOI: 10.1038/nrm3025]

21   **Sarbassov DD**, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, Markhard AL, Sabatini DM. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell* 2006; **22**: 159-168 [PMID: 16603397 DOI: 10.1016/j.molcel.2006.03.029]

22   **Zinzalla V**, Stracka D, Oppliger W, Hall MN. Activation of mTORC2 by association with the ribosome. *Cell* 2011; **144**: 757-768 [PMID: 21376236 DOI: 10.1016/j.cell.2011.02.014]

23   **Villanueva A**, Chiang DY, Newell P, Peix J, Thung S, Alsinet C, Tovar V, Roayaie S, Minguez B, Sole M, Battiston C, Van Laarhoven S, Fiel MI, Di Feo A, Hoshida Y, Yea S, Toffanin S, Ramos A, Martignetti JA, Mazzaferro V, Bruix J, Waxman S, Schwartz M, Meyerson M, Friedman SL, Llovet JM. Pivotal role of mTOR signaling in hepatocellular carcinoma. *Gastroenterology* 2008; **135**: 1972-1983, 1983.e1-1983.11 [PMID: 18929564 DOI: 10.1053/j.gastro.2008.08.008]

24   **Jiang D**, Cho W, Li Z, Xu X, Qu Y, Jiang Z, Guo L, Xu G. MiR-758-3p suppresses proliferation, migration and invasion of hepatocellular carcinoma cells via targeting MDM2 and mTOR. *Biomed Pharmacother* 2017; **96**: 535-544 [PMID: 29032337 DOI: 10.1016/j.biopha.2017.10.004]

25   **Yu Q**, Xiang L, Yin L, Liu X, Yang D, Zhou J. Loss-of-function of miR-142 by hypermethylation promotes TGF-β-mediated tumour growth and metastasis in hepatocellular carcinoma. *Cell Prolif* 2017; **50**: [PMID: 28963738 DOI: 10.1111/cpr.12384]

26   **Zhou SJ**, Liu FY, Zhang AH, Liang HF, Wang Y, Ma R, Jiang YH, Sun NF. MicroRNA-199b-5p attenuates TGF-β1-induced epithelial-mesenchymal transition in hepatocellular carcinoma. *Br J Cancer* 2017; **117**: 233-244 [PMID: 28588321 DOI: 10.1038/bjc.2017.164]

27   **Tovar V**, Alsinet C, Villanueva A, Hoshida Y, Chiang DY, Solé M, Thung S, Moyano S, Toffanin S, Mínguez B, Cabellos L, Peix J, Schwartz M, Mazzaferro V, Bruix J, Llovet JM. IGF activation in a molecular subclass of hepatocellular carcinoma and pre-clinical efficacy of IGF-1R blockage. *J Hepatol* 2010; **52**: 550-559 [PMID: 20206398 DOI: 10.1016/j.jhep.2010.01.015]

28   **Cheng H**, Xue J, Yang S, Chen Y, Wang Y, Zhu Y, Wang X, Kuang D,

Ruan Q, Duan Y, Wang G. Co-targeting of IGF1R/mTOR pathway by miR-497 and miR-99a impairs hepatocellular carcinoma development. *Oncotarget* 2017; **8**: 47984-47997 [PMID: 28624790 DOI: 10.18632/oncotarget.18207]

29   **Han X**, Wang X, Zhao B, Chen G, Sheng Y, Wang W, Teng M. MicroRNA-187 inhibits tumor growth and metastasis via targeting of IGF-1R in hepatocellular carcinoma. *Mol Med Rep* 2017; **16**: 2241-2246 [PMID: 28627639 DOI: 10.3892/mmr.2017.6788]

30   **Wang W**, Zhang H, Tang M, Liu L, Zhou Z, Zhang S, Wang L. MicroRNA-592 targets IGF-1R to suppress cellular proliferation, migration and invasion in hepatocellular carcinoma. *Oncol Lett* 2017; **13**: 3522-3528 [PMID: 28529580 DOI: 10.3892/ol.2017.5902]

31   **Ma X**, Zhuang B, Li W. MicroRNA-296-5p downregulated AKT2 to inhibit hepatocellular carcinoma cell proliferation, migration and invasion. *Mol Med Rep* 2017; **16**: 1565-1572 [PMID: 28586057 DOI: 10.3892/mmr.2017.6701]

32   **Mo Y**, Lu Y, Wang P, Huang S, He L, Li D, Li F, Huang J, Lin X, Li X, Che S, Chen Q. Long non-coding RNA XIST promotes cell growth by regulating miR-139-5p/PDK1/AKT axis in hepatocellular carcinoma. *Tumour Biol* 2017; **39**: 1010428317690999 [PMID: 28231734 DOI: 10.1177/1010428317690999]

33   **Li H**, Zhang J, Lee MJ, Yu GR, Han X, Kim DG. OIP5, a target of miR-15b-5p, regulates hepatocellular carcinoma growth and metastasis through the AKT/mTORC1 and β-catenin signaling pathways. *Oncotarget* 2017; **8**: 18129-18144 [PMID: 28184024 DOI: 10.18632/oncotarget.15185]

34   **Yu M**, Xue H, Wang Y, Shen Q, Jiang Q, Zhang X, Li K, Jia M, Jia J, Xu J, Tian Y. miR-345 inhibits tumor metastasis and EMT by targeting IRF1-mediated mTOR/STAT3/AKT pathway in hepatocellular carcinoma. *Int J Oncol* 2017; **50**: 975-983 [PMID: 28098858 DOI: 10.3892/ijo.2017.3852]

35   **Dong Z**, Qi R, Guo X, Zhao X, Li Y, Zeng Z, Bai W, Chang X, Hao L, Chen Y, Lou M, Li Z, Lu Y. MiR-223 modulates hepatocellular carcinoma cell proliferation through promoting apoptosis via the Rab1-mediated mTOR activation. *Biochem Biophys Res Commun* 2017; **483**: 630-637 [PMID: 27998765 DOI: 10.1016/j.bbrc.2016.12.091]

36   **Chang W**, Zhang L, Xian Y, Yu Z. MicroRNA-33a promotes cell proliferation and inhibits apoptosis by targeting PPARα in human hepatocellular carcinoma. *Exp Ther Med* 2017; **13**: 2507-2514 [PMID: 28565872 DOI: 10.3892/etm.2017.4236]

37   **Chen YL**, Xu QP, Guo F, Guan WH. MicroRNA-302d downregulates TGFBR2 expression and promotes hepatocellular carcinoma growth and invasion. *Exp Ther Med* 2017; **13**: 681-687 [PMID: 28352351 DOI: 10.3892/etm.2016.3970]

38   **Zhuang L**, Wang X, Wang Z, Ma X, Han B, Zou H, Wu Z, Dong S, Qu Z, Zang Y, Wu L. MicroRNA-23b functions as an oncogene and activates AKT/GSK3β/β-catenin signaling by targeting ST7L in hepatocellular carcinoma. *Cell Death Dis* 2017; **8**: e2804 [PMID: 28518144 DOI: 10.1038/cddis.2017.216]

39   **Chang S**, Chen B, Wang X, Wu K, Sun Y. Long non-coding RNA XIST regulates PTEN expression by sponging miR-181a and promotes hepatocellular carcinoma progression. *BMC Cancer* 2017; **17**: 248 [PMID: 28388883 DOI: 10.1186/s12885-017-3216-6]

40   **Fu X**, Wen H, Jing L, Yang Y, Wang W, Liang X, Nan K, Yao Y, Tian T. MicroRNA-155-5p promotes hepatocellular carcinoma progression by suppressing PTEN through the PI3K/Akt pathway. *Cancer Sci* 2017; **108**: 620-631 [PMID: 28132399 DOI: 10.1111/cas.13177]

41   **Feng X**, Jiang J, Shi S, Xie H, Zhou L, Zheng S. Knockdown of miR-25 increases the sensitivity of liver cancer stem cells to TRAIL-induced apoptosis via PTEN/PI3K/Akt/Bad signaling pathway. *Int J Oncol* 2016; **49**: 2600-2610 [PMID: 27840896 DOI: 10.3892/ijo.2016.3751]

42   **Gordon MD**, Nusse R. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. *J Biol Chem* 2006; **281**: 22429-22433 [PMID: 16793760 DOI: 10.1074/jbc.R600015200]

43   **Komiya Y**, Habas R. Wnt signal transduction pathways. *Organogenesis* 2008; **4**: 68-75 [PMID: 19279717]

44   **MacDonald BT**, Tamai K, He X. Wnt/beta-catenin signaling:

components, mechanisms, and diseases. *Dev Cell* 2009; **17**: 9-26 [PMID: 19619488 DOI: 10.1016/j.devcel.2009.06.016]

45   **Polakis P**. Wnt signaling in cancer. *Cold Spring Harb Perspect Biol* 2012; **4** pii: a008052 [PMID: 22438566 DOI: 10.1101/cshperspect.a008052]

46   **Zeng G**, Apte U, Cieply B, Singh S, Monga SP. siRNA-mediated beta-catenin knockdown in human hepatoma cells results in decreased growth and survival. *Neoplasia* 2007; **9**: 951-959 [PMID: 18030363]

47   **Ashmawy AM**, Elgeshy KM, Abdel Salam ET, Ghareeb M, Kobaisi MH, Amin HAA, Sharawy SK, Abdel Wahab AHA. Crosstalk between liver-related microRNAs and Wnt/β-catenin pathway in hepatocellular carcinoma patients. *Arab J Gastroenterol* 2017; **18**: 144-150 [PMID: 28958640 DOI: 10.1016/j.ajg.2017.09.001]

48   **Liu L**, Cai X, Liu E, Tian X, Tian C. MicroRNA-18a promotes proliferation and metastasis in hepatocellular carcinoma via targeting KLF4. *Oncotarget* 2017; **8**: 68263-68269 [PMID: 28978114 DOI: 10.18632/oncotarget.19293]

49   **Sarkar D**, Fisher PB. AEG-1/MTDH/LYRIC: clinical significance. *Adv Cancer Res* 2013; **120**: 39-74 [PMID: 23889987 DOI: 10.1016/B978-0-12-401676-7.00002-4]

50   **Yan JJ**, Chang Y, Zhang YN, Lin JS, He XX, Huang HJ. miR-195 inhibits cell proliferation via targeting AEG-1 in hepatocellular carcinoma. *Oncol Lett* 2017; **13**: 3118-3126 [PMID: 28529562 DOI: 10.3892/ol.2017.5826]

51   **Lu C**, Liao Z, Cai M, Zhang G. MicroRNA-320a downregulation mediates human liver cancer cell proliferation through the Wnt/β-catenin signaling pathway. *Oncol Lett* 2017; **13**: 573-578 [PMID: 28356931 DOI: 10.3892/ol.2016.5479]

52   **Xie F**, Yuan Y, Xie L, Ran P, Xiang X, Huang Q, Qi G, Guo X, Xiao C, Zheng S. miRNA-320a inhibits tumor proliferation and invasion by targeting c-Myc in human hepatocellular carcinoma. *Onco Targets Ther* 2017; **10**: 885-894 [PMID: 28243124 DOI: 10.2147/OTT.S122992]

53   **Jiang T**, Li M, Li Q, Guo Z, Sun X, Zhang X, Liu Y, Yao W, Xiao P. MicroRNA-98-5p Inhibits Cell Proliferation and Induces Cell Apoptosis in Hepatocellular Carcinoma via Targeting IGF2BP1. *Oncol Res* 2017; **25**: 1117-1127 [PMID: 28244848 DOI: 10.3727/096504016X14821952695683]

54   **Chu Y**, Fan W, Guo W, Zhang Y, Wang L, Guo L, Duan X, Wei J, Xu G. miR-1247-5p functions as a tumor suppressor in human hepatocellular carcinoma by targeting Wnt3. *Oncol Rep* 2017; **38**: 343-351 [PMID: 28586038 DOI: 10.3892/or.2017.5702]

55   **Haddad BR**, Gu L, Mirtti T, Dagvadorj A, Vogiatzi P, Hoang DT, Bajaj R, Leiby B, Ellsworth E, Blackmon S, Ruiz C, Curtis M, Fortina P, Ertel A, Liu C, Rui H, Visakorpi T, Bubendorf L, Lallas CD, Trabulsi EJ, McCue P, Gomella L, Nevalainen MT. STAT5A/B gene locus undergoes amplification during human prostate cancer progression. *Am J Pathol* 2013; **182**: 2264-2275 [PMID: 23660011 DOI: 10.1016/j.ajpath.2013.02.044]

56   **He B**, You L, Uematsu K, Zang K, Xu Z, Lee AY, Costello JF, McCormick F, Jablons DM. SOCS-3 is frequently silenced by hypermethylation and suppresses cell growth in human lung cancer. *Proc Natl Acad Sci U S A* 2003; **100**: 14133-14138 [PMID: 14617776 DOI: 10.1073/pnas.2232790100]

57   **Thomas SJ**, Snowden JA, Zeidler MP, Danson SJ. The role of JAK/STAT signalling in the pathogenesis, prognosis and treatment of solid tumours. *Br J Cancer* 2015; **113**: 365-371 [PMID: 26151455 DOI: 10.1038/bjc.2015.233]

58   **Huang PS**, Lin YH, Chi HC, Chen PY, Huang YH, Yeh CT, Wang CS, Lin KH. Thyroid hormone inhibits growth of hepatoma cells through induction of miR-214. *Sci Rep* 2017; **7**: 14868 [PMID: 29093516 DOI: 10.1038/s41598-017-14864-1]

59   **Xie Y**, Xu K, Dai B, Guo Z, Jiang T, Chen H, Qiu Y. The 44 kDa Pim-1 kinase directly interacts with tyrosine kinase Etk/BMX and protects human prostate cancer cells from apoptosis induced by chemotherapeutic drugs. *Oncogene* 2006; **25**: 70-78 [PMID: 16186805 DOI: 10.1038/sj.onc.1209058]

60   **Mao J**, Hu X, Pang P, Zhou B, Li D, Shan H. miR-30e acts as a tumor suppressor in hepatocellular carcinoma partly via JAK1/ STAT3 pathway. *Oncol Rep* 2017; **38**: 393-401 [PMID: 28560434 DOI: 10.3892/or.2017.5683]

61   **Yuan J**, Ji H, Xiao F, Lin Z, Zhao X, Wang Z, Zhao J, Lu J. MicroRNA-340 inhibits the proliferation and invasion of hepatocellular carcinoma cells by targeting JAK1. *Biochem Biophys Res Commun* 2017; **483**: 578-584 [PMID: 27998770 DOI: 10.1016/j.bbrc.2016.12.102]

62   **Yan X**, Zhu Z, Xu S, Yang LN, Liao XH, Zheng M, Yang D, Wang J, Chen D, Wang L, Liu X, Liu J, Chen RH, Zhou XZ, Lu KP, Liu H. MicroRNA-140-5p inhibits hepatocellular carcinoma by directly targeting the unique isomerase Pin1 to block multiple cancer-driving pathways. *Sci Rep* 2017; **7**: 45915 [PMID: 28383568 DOI: 10.1038/srep45915]

63   **Pang R**, Yuen J, Yuen MF, Lai CL, Lee TK, Man K, Poon RT, Fan ST, Wong CM, Ng IO, Kwong YL, Tse E. PIN1 overexpression and beta-catenin gene mutations are distinct oncogenic events in human hepatocellular carcinoma. *Oncogene* 2004; **23**: 4182-4186 [PMID: 15064734 DOI: 10.1038/sj.onc.1207493]

64   **Shinoda K**, Kuboki S, Shimizu H, Ohtsuka M, Kato A, Yoshitomi H, Furukawa K, Miyazaki M. Pin1 facilitates NF-κB activation and promotes tumour progression in human hepatocellular carcinoma. *Br J Cancer* 2015; **113**: 1323-1331 [PMID: 26461058 DOI: 10.1038/bjc.2015.272]

65   **Huang P**, Qiu J, Li B, Hong J, Lu C, Wang L, Wang J, Hu Y, Jia W, Yuan Y. Role of Sox2 and Oct4 in predicting survival of hepatocellular carcinoma patients after hepatectomy. *Clin Biochem* 2011; **44**: 582-589 [PMID: 21376027 DOI: 10.1016/j.clinbiochem.2011.02.012]

66   **Wen W**, Han T, Chen C, Huang L, Sun W, Wang X, Chen SZ, Xiang DM, Tang L, Cao D, Feng GS, Wu MC, Ding J, Wang HY. Cyclin G1 expands liver tumor-initiating cells by Sox2 induction via Akt/mTOR signaling. *Mol Cancer Ther* 2013; **12**: 1796-1804 [PMID: 23804702 DOI: 10.1158/1535-7163.MCT-13-0099]

67   **Sun C**, Sun L, Li Y, Kang X, Zhang S, Liu Y. Sox2 expression predicts poor survival of hepatocellular carcinoma patients and it promotes liver cancer cell invasion by activating Slug. *Med Oncol* 2013; **30**: 503 [PMID: 23430442 DOI: 10.1007/s12032-013-0503-1]

68   **Zhang Y**, Zhang D, Jiang J, Dong L. Loss of miR-638 promotes invasion and epithelial-mesenchymal transition by targeting SOX2 in hepatocellular carcinoma. *Oncol Rep* 2017; **37**: 323-332 [PMID: 27878280 DOI: 10.3892/or.2016.5273]

69   **Elmore S**. Apoptosis: a review of programmed cell death. *Toxicol Pathol* 2007; **35**: 495-516 [PMID: 17562483 DOI: 10.1080/01926230701320337]

70   **Norbury CJ**, Hickson ID. Cellular responses to DNA damage. *Annu Rev Pharmacol Toxicol* 2001; **41**: 367-401 [PMID: 11264462 DOI: 10.1146/annurev.pharmtox.41.1.367]

71   **Wallace-Brodeur RR**, Lowe SW. Clinical implications of p53 mutations. *Cell Mol Life Sci* 1999; **55**: 64-75 [PMID: 10065152 DOI: 10.1007/s000180050270]

72   **Lowe SW**, Lin AW. Apoptosis in cancer. *Carcinogenesis* 2000; **21**: 485-495 [PMID: 10688869]

73   **Fu XT**, Shi YH, Zhou J, Peng YF, Liu WR, Shi GM, Gao Q, Wang XY, Song K, Fan J, Ding ZB. MicroRNA-30a suppresses autophagy-mediated anoikis resistance and metastasis in hepatocellular carcinoma. *Cancer Lett* 2018; **412**: 108-117 [PMID: 29061507 DOI: 10.1016/j.canlet.2017.10.012]

74   **Li M**, Yang Y, Kuang Y, Gan X, Zeng W, Liu Y, Guan H. miR-365 induces hepatocellular carcinoma cell apoptosis through targeting Bcl-2. *Exp Ther Med* 2017; **13**: 2279-2285 [PMID: 28565839 DOI: 10.3892/etm.2017.4244]

75   **Zhan L**, Pan Y, Chen L, Chen Z, Zhang H, Sun C. MicroRNA-526a targets p21-activated kinase 7 to inhibit tumorigenesis in hepatocellular carcinoma. *Mol Med Rep* 2017; **16**: 837-844 [PMID: 28560394 DOI: 10.3892/mmr.2017.6658]

76   **Shigematsu S**, Fukuda S, Nakayama H, Inoue H, Hiasa Y, Onji M, Higashiyama S. ZNF689 suppresses apoptosis of hepatocellular carcinoma cells through the down-regulation of Bcl-2 family members. *Exp Cell Res* 2011; **317**: 1851-1859 [PMID: 21624362 DOI: 10.1016/j.yexcr.2011.05.012]

77 **Ge H**, Zou D, Wang Y, Jiang H, Wang L. MicroRNA-377 Downregulates Bcl-xL and Increases Apoptosis in Hepatocellular Carcinoma Cells. *Oncol Res* 2017; **25**: 29-34 [PMID: 28081730 DOI: 10.3727/096504016X14719078133168]

78 **Liu L**, Lu L, Zheng A, Xie J, Xue Q, Wang F, Wang X, Zhou H, Tong X, Li Y, Zhu X, Wu G. MiR-199a-5p and let-7c cooperatively inhibit migration and invasion by targeting MAP4K3 in hepatocellular carcinoma. *Oncotarget* 2017; **8**: 13666-13677 [PMID: 28099144 DOI: 10.18632/oncotarget.14623]

79 **Zhu XM**, Wu LJ, Xu J, Yang R, Wu FS. Let-7c microRNA expression and clinical significance in hepatocellular carcinoma. *J Int Med Res* 2011; **39**: 2323-2329 [PMID: 22289550 DOI: 10.1177/147323001103900631]

80 **Lam D**, Dickens D, Reid EB, Loh SH, Moisoi N, Martins LM. MAP4K3 modulates cell death via the post-transcriptional regulation of BH3-only proteins. *Proc Natl Acad Sci U S A* 2009; **106**: 11978-11983 [PMID: 19587239 DOI: 10.1073/pnas.0900608106]

81 **Li M**, Zhu Y, Zhang H, Li L, He P, Xia H, Zhang Y, Mao C. Delivery of inhibitor of growth 4 (ING4) gene significantly inhibits proliferation and invasion and promotes apoptosis of human osteosarcoma cells. *Sci Rep* 2014; **4**: 7380 [PMID: 25490312 DOI: 10.1038/srep07380]

82 **Hu X**, Feng Y, Sun L, Qu L, Sun C. Roles of microRNA-330 and Its Target Gene ING4 in the Development of Aggressive Phenotype in Hepatocellular Carcinoma Cells. *Dig Dis Sci* 2017; **62**: 715-722 [PMID: 28050784 DOI: 10.1007/s10620-016-4429-2]

83 **Dhillon AS**, Hagan S, Rath O, Kolch W. MAP kinase signalling pathways in cancer. *Oncogene* 2007; **26**: 3279-3290 [PMID: 17496922 DOI: 10.1038/sj.onc.1210421]

84 **Sarris ME**, Moulos P, Haroniti A, Giakountis A, Talianidis I. Smyd3 Is a Transcriptional Potentiator of Multiple Cancer-Promoting Genes and Required for Liver and Colon Cancer Development. *Cancer Cell* 2016; **29**: 354-366 [PMID: 26908355 DOI: 10.1016/j.ccell.2016.01.013]

85 **Mazur PK**, Reynoird N, Khatri P, Jansen PW, Wilkinson AW, Liu S, Barbash O, Van Aller GS, Huddleston M, Dhanak D, Tummino PJ, Kruger RG, Garcia BA, Butte AJ, Vermeulen M, Sage J, Gozani O. SMYD3 links lysine methylation of MAP3K2 to Ras-driven cancer. *Nature* 2014; **510**: 283-287 [PMID: 24847881 DOI: 10.1038/nature13320]

86 **Zhu W**, Qian J, Ma L, Ma P, Yang F, Shu Y. MiR-346 suppresses cell proliferation through SMYD3 dependent approach in hepatocellular carcinoma. *Oncotarget* 2017; **8**: 65218-65229 [PMID: 29029425 DOI: 10.18632/oncotarget.18060]

87 **Xue F**, Yin J, Xu L, Wang B. MicroRNA-143 inhibits tumorigenesis in hepatocellular carcinoma by downregulating GATA6. *Exp Ther Med* 2017; **13**: 2667-2674 [PMID: 28587328 DOI: 10.3892/etm.2017.4348]

88 **Kwei KA**, Bashyam MD, Kao J, Ratheesh R, Reddy EC, Kim YH, Montgomery K, Giacomini CP, Choi YL, Chatterjee S, Karikari CA, Salari K, Wang P, Hernandez-Boussard T, Swarnalata G, van de Rijn M, Maitra A, Pollack JR. Genomic profiling identifies GATA6 as a candidate oncogene amplified in pancreatobiliary cancer. *PLoS Genet* 2008; **4**: e1000081 [PMID: 18535672 DOI: 10.1371/journal.pgen.1000081]

89 **Coppola N**, de Stefano G, Panella M, Onorato L, Iodice V, Minichini C, Mosca N, Desiato L, Farella N, Starace M, Liorre G, Potenza N, Sagnelli E, Russo A. Lowered expression of microRNA-125a-5p in human hepatocellular carcinoma and up-regulation of its oncogenic targets sirtuin-7, matrix metalloproteinase-11, and c-Raf. *Oncotarget* 2017; **8**: 25289-25299 [PMID: 28445974 DOI: 10.18632/oncotarget.15809]

90 **Shi X**, Wang X. The role of MTDH/AEG-1 in the progression of cancer. *Int J Clin Exp Med* 2015; **8**: 4795-4807 [PMID: 26131054]

91 **Zhang M**, Li M, Li N, Zhang Z, Liu N, Han X, Liu Q, Liao C. miR-217 suppresses proliferation, migration, and invasion promoting apoptosis via targeting MTDH in hepatocellular carcinoma. *Oncol Rep* 2017; **37**: 1772-1778 [PMID: 28184926 DOI: 10.3892/or.2017.5401]

92 **Wang J**, Song W, Shen W, Yang X, Sun W, Qu S, Shang R, Ma B, Pu M, Tao K, Dou K, Li H. MicroRNA-200a Suppresses Cell Invasion and Migration by Directly Targeting GAB1 in Hepatocellular Carcinoma. *Oncol Res* 2017; **25**: 1-10 [PMID: 28081727 DOI: 10.3727/096504016X14685034103798]

93 **Sato F**, Hatano E, Kitamura K, Myomoto A, Fujiwara T, Takizawa S, Tsuchiya S, Tsujimoto G, Uemoto S, Shimizu K. MicroRNA profile predicts recurrence after resection in patients with hepatocellular carcinoma within the Milan Criteria. *PLoS One* 2011; **6**: e16435 [PMID: 21298008 DOI: 10.1371/journal.pone.0016435]

94 **Shimagaki T**, Yoshizumi T, Harimoto N, Yoshio S, Naito Y, Yamamoto Y, Ochiya T, Yoshida Y, Kanto T, Maehara Y. MicroRNA-125b expression and intrahepatic metastasis are predictors for early recurrence after hepatocellular carcinoma resection. *Hepatol Res* 2018; **48**: 313-321 [PMID: 28984009 DOI: 10.1111/hepr.12990]

95 **Gao Y**, Zhang SG, Wang ZH, Liao JC. Down-regulation of miR-342-3p in hepatocellular carcinoma tissues and its prognostic significance. *Eur Rev Med Pharmacol Sci* 2017; **21**: 2098-2102 [PMID: 28537676]

96 **Zhao XQ**, Liang B, Jiang K, Zhang HY. Down-regulation of miR-655-3p predicts worse clinical outcome in patients suffering from hepatocellular carcinoma. *Eur Rev Med Pharmacol Sci* 2017; **21**: 748-752 [PMID: 28272708]

97 **Ma YS**, Wu TM, Lv ZW, Lu GX, Cong XL, Xie RT, Yang HQ, Chang ZY, Sun R, Chai L, Cai MX, Zhong XJ, Zhu J, Fu D. High expression of miR-105-1 positively correlates with clinical prognosis of hepatocellular carcinoma by targeting oncogene NCOA1. *Oncotarget* 2017; **8**: 11896-11905 [PMID: 28060733 DOI: 10.18632/oncotarget.14435]

98 **Imura S**, Yamada S, Saito YU, Iwahashi S, Arakawa Y, Ikemoto T, Morine Y, Utsunomiya T, Shimada M. miR-223 and Stathmin-1 Expression in Non-tumor Liver Tissue of Patients with Hepatocellular Carcinoma. *Anticancer Res* 2017; **37**: 5877-5883 [PMID: 28982915 DOI: 10.21873/anticanres.12033]

99 **Vasuri F**, Fittipaldi S, De Pace V, Gramantieri L, Bertuzzo V, Cescon M, Pinna AD, Fiorentino M, D'Errico A, Ravaioli M. Tissue miRNA 483-3p expression predicts tumor recurrence after surgical resection in histologically advanced hepatocellular carcinomas. *Oncotarget* 2018; **9**: 17895-17905 [PMID: 29707155 DOI: 10.18632/oncotarget.24860]

100 **Hung CL**, Yen CS, Tsai HW, Su YC, Yen CJ. Upregulation of MicroRNA-19b predicts good prognosis in patients with hepatocellular carcinoma presenting with vascular invasion or multifocal disease. *BMC Cancer* 2015; **15**: 665 [PMID: 26453548 DOI: 10.1186/s12885-015-1671-5]

101 **Chen F**, Li XF, Fu DS, Huang JG, Yang SE. Clinical potential of miRNA-221 as a novel prognostic biomarker for hepatocellular carcinoma. *Cancer Biomark* 2017; **18**: 209-214 [PMID: 27983537 DOI: 10.3233/CBM-161671]

102 **von Felden J**, Heim D, Schulze K, Krech T, Ewald F, Nashan B, Lohse AW, Wege H. High expression of micro RNA-135A in hepatocellular carcinoma is associated with recurrence within 12 months after resection. *BMC Cancer* 2017; **17**: 60 [PMID: 28100188 DOI: 10.1186/s12885-017-3053-7]

103 **Zhu HT**, Dong QZ, Sheng YY, Wei JW, Wang G, Zhou HJ, Ren N, Jia HL, Ye QH, Qin LX. MicroRNA-29a-5p is a novel predictor for early recurrence of hepatitis B virus-related hepatocellular carcinoma after surgical resection. *PLoS One* 2012; **7**: e52393 [PMID: 23285022 DOI: 10.1371/journal.pone.0052393]

104 **Barry CT**, D'Souza M, McCall M, Safadjou S, Ryan C, Kashyap R, Marroquin C, Orloff M, Almudevar A, Godfrey TE. Micro RNA expression profiles as adjunctive data to assess the risk of hepatocellular carcinoma recurrence after liver transplantation. *Am J Transplant* 2012; **12**: 428-437 [PMID: 22008552 DOI: 10.1111/j.1600-6143.2011.03788.x]

105 **Morita K**, Shirabe K, Taketomi A, Soejima Y, Yoshizumi T, Uchiyama H, Ikegami T, Yamashita Y, Sugimachi K, Harimoto N, Itoh S, Ikeda T, Maehara Y. Relevance of microRNA-18a and microRNA-199a-5p to hepatocellular carcinoma recurrence after

living donor liver transplantation. *Liver Transpl* 2016; **22**: 665-676 [PMID: 26783726 DOI: 10.1002/lt.24400]

106   **Lu AQ**, Lv B, Qiu F, Wang XY, Cao XH. Upregulation of miR-137 reverses sorafenib resistance and cancer-initiating cell phenotypes by degrading ANT2 in hepatocellular carcinoma. *Oncol Rep* 2017; **37**: 2071-2078 [PMID: 28350139 DOI: 10.3892/or.2017.5498]

107   **Potenza N**, Mosca N, Zappavigna S, Castiello F, Panella M, Ferri C, Vanacore D, Giordano A, Stiuso P, Caraglia M, Russo A. MicroRNA-125a-5p Is a Downstream Effector of Sorafenib in Its Antiproliferative Activity Toward Human Hepatocellular Carcinoma Cells. *J Cell Physiol* 2017; **232**: 1907-1913 [PMID: 27982429 DOI: 10.1002/jcp.25744]

108   **Nishida N**, Arizumi T, Hagiwara S, Ida H, Sakurai T, Kudo M. MicroRNAs for the Prediction of Early Response to Sorafenib Treatment in Human Hepatocellular Carcinoma. *Liver Cancer* 2017; **6**: 113-125 [PMID: 28275578 DOI: 10.1159/000449475]

109   **Shao P**, Qu WK, Wang CY, Tian Y, Ye ML, Sun DG, Sui JD, Wang LM, Fan R, Gao ZM. MicroRNA-205-5p regulates the chemotherapeutic resistance of hepatocellular carcinoma cells by targeting PTEN/JNK/ANXA3 pathway. *Am J Transl Res* 2017; **9**: 4300-4307 [PMID: 28979703]

110   **Yang X**, Zang J, Pan X, Yin J, Xiang Q, Yu J, Gan R, Lei X. miR-503 inhibits proliferation making human hepatocellular carcinoma cells susceptible to 5-fluorouracil by targeting EIF4E. *Oncol Rep* 2017; **37**: 563-570 [PMID: 27840964 DOI: 10.3892/or.2016.5220]

111   **Meng W**, Tai Y, Zhao H, Fu B, Zhang T, Liu W, Li H, Yang Y, Zhang Q, Feng Y, Chen G. Downregulation of miR-33a-5p in Hepatocellular Carcinoma: A Possible Mechanism for Chemotherapy Resistance. *Med Sci Monit* 2017; **23**: 1295-1304 [PMID: 28291769]

112   **Du Z**, Niu S, Xu X, Xu Q. MicroRNA31-NDRG3 regulation axes are essential for hepatocellular carcinoma survival and drug resistance. *Cancer Biomark* 2017; **19**: 221-230 [PMID: 28269758 DOI: 10.3233/CBM-170568]

113   **Jin F**, Wang Y, Li M, Zhu Y, Liang H, Wang C, Wang F, Zhang CY, Zen K, Li L. MiR-26 enhances chemosensitivity and promotes apoptosis of hepatocellular carcinoma cells through inhibiting autophagy. *Cell Death Dis* 2017; **8**: e2540 [PMID: 28079894 DOI: 10.1038/cddis.2016.461]

114   **Loosen SH**, Schueller F, Trautwein C, Roy S, Roderburg C. Role of circulating microRNAs in liver diseases. *World J Hepatol* 2017; **9**: 586-594 [PMID: 28515844 DOI: 10.4254/wjh.v9.i12.586]

115   **Xu J**, Wu C, Che X, Wang L, Yu D, Zhang T, Huang L, Li H, Tan W, Wang C, Lin D. Circulating microRNAs, miR-21, miR-122, and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. *Mol Carcinog* 2011; **50**: 136-142 [PMID: 21229610 DOI: 10.1002/mc.20712]

116   **Ding Y**, Yan JL, Fang AN, Zhou WF, Huang L. Circulating miRNAs as novel diagnostic biomarkers in hepatocellular carcinoma detection: a meta-analysis based on 24 articles. *Oncotarget* 2017; **8**: 66402-66413 [PMID: 29029522 DOI: 10.18632/oncotarget.18949]

117   **Zhang Y**, Li T, Qiu Y, Zhang T, Guo P, Ma X, Wei Q, Han L. Serum microRNA panel for early diagnosis of the onset of hepatocellular carcinoma. *Medicine* (Baltimore) 2017; **96**: e5642 [PMID: 28079796 DOI: 10.1097/MD.0000000000005642]

118   **Fornari F**, Ferracin M, Trerè D, Milazzo M, Marinelli S, Galassi M, Venerandi L, Pollutri D, Patrizi C, Borghi A, Foschi FG, Stefanini GF, Negrini M, Bolondi L, Gramantieri L. Circulating microRNAs, miR-939, miR-595, miR-519d and miR-494, Identify Cirrhotic Patients with HCC. *PLoS One* 2015; **10**: e0141448 [PMID: 26509672 DOI: 10.1371/journal.pone.0141448]

119   **Wang L**, Liu M, Zhu H, Rong W, Wu F, An S, Liu F, Feng L, Wu J, Xu N. Identification of recurrence-related serum microRNAs in hepatocellular carcinoma following hepatectomy. *Cancer Biol Ther* 2015; **16**: 1445-1452 [PMID: 26176380 DOI: 10.1080/15384047.2015.1071730]

120   **Xiang ZL**, Zhao XM, Zhang L, Yang P, Fan J, Tang ZY, Zeng ZC. MicroRNA-34a expression levels in serum and intratumoral tissue can predict bone metastasis in patients with hepatocellular carcinoma. *Oncotarget* 2016; **7**: 87246-87256 [PMID: 27893432 DOI: 10.18632/oncotarget.13531]

121   **Fittipaldi S**, Vasuri F, Bonora S, Degiovanni A, Santandrea G, Cucchetti A, Gramantieri L, Bolondi L, D'Errico A. miRNA Signature of Hepatocellular Carcinoma Vascularization: How the Controls Can Influence the Signature. *Dig Dis Sci* 2017; **62**: 2397-2407 [PMID: 28639131 DOI: 10.1007/s10620-017-4654-3]

122   **Shen J**, Wang Q, Gurvich I, Remotti H, Santella RM. Evaluating normalization approaches for the better identification of aberrant microRNAs associated with hepatocellular carcinoma. *Hepatoma Res* 2016; **2**: 305-315 [PMID: 28393113 DOI: 10.20517/2394-5079.2016.28]

REVIEW

Apoptosis and non-alcoholic fatty liver diseases

Tatsuo Kanda, Shunichi Matsuoka, Motomi Yamazaki, Toshikatsu Shibata, Kazushige Nirei, Hiroshi Takahashi, Tomohiro Kaneko, Mariko Fujisawa, Teruhisa Higuchi, Hitomi Nakamura, Naoki Matsumoto, Hiroaki Yamagami, Masahiro Ogawa, Hiroo Imazu, Kazumichi Kuroda, Mitsuhiko Moriyama

Tatsuo Kanda, Shunichi Matsuoka, Motomi Yamazaki, Toshikatsu Shibata, Kazushige Nirei, Hiroshi Takahashi, Tomohiro Kaneko, Mariko Fujisawa, Teruhisa Higuchi, Hitomi Nakamura, Naoki Matsumoto, Hiroaki Yamagami, Masahiro Ogawa, Hiroo Imazu, Kazumichi Kuroda, Mitsuhiko Moriyama, Division of Gastroenterology and Hepatology, Department of Medicine, Nihon University School of Medicine, Itabashi-ku, Tokyo 173-8610, Japan

ORCID number: Tatsuo Kanda (0000-0002-9565-4669); Shunichi Matsuoka (0000-0002-3221-7939); Motomi Yamazaki (0000-0002-4890-846X); Toshikatsu Shibata (0000-0003-3235-4893); Kazushige Nirei (0000-0003-3926-5076); Hiroshi Takahashi (0000-0003-4847-438X); Tomohiro Kaneko (0000-0002-1726-5852); Mariko Fujisawa (0000-0003-0843-3065); Teruhisa Higuchi (0000-0003-4104-0213); Hitomi Nakamura (0000-0001-7748-5833); Naoki Matsumoto (0000-0002-9982-6130); Hiroaki Yamagami (0000-0003-0174-7811); Masahiro Ogawa (0000-0003-2154-7999); Hiroo Imazu (0000-0003-4038-7919); Kazumichi Kuroda (0000-0002-9557-1046); Mitsuhiko Moriyama (0000-0002-4617-508X).

Author contributions: Kanda T designed research, performed research, and analyzed data; Kanda T, Matsuoka S, Yamazaki M, Shibata T, Nirei K, Takahashi H, Kaneko T, Fujisawa M, Higuchi T, Nakamura H, Matsumoto N, Yamagami H, Ogawa M, Imazu H, Kuroda K, and Moriyama M wrote the paper.

Conflict-of-interest statement: Kanda T received research grants from AbbVie, MSD, Chugai Pharma, and Sysmex. These companies played no role in this study. Other authors declare no conflict of interests related to this publication.

Correspondence to: Tatsuo Kanda, MD, PhD, Associate Professor, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Nihon University School of Medicine, 30-1 Oyaguchi-Kamicho, Itabashi-ku, Tokyo 173-8610, Japan. kanda2t @yahoo.co.jp
Telephone: +81-3-39728111
Fax: +81-3-39568496

Abstract

The number of patients with nonalcoholic fatty liver diseases (NAFLD) including nonalcoholic steatohepatitis (NASH), has been increasing. NASH causes cirrhosis and hepatocellular carcinoma (HCC) and is one of the most serious health problems in the world. The mechanism through which NASH progresses is still largely unknown. Activation of caspases, Bcl-2 family proteins, and c-Jun N-terminal kinase-induced hepatocyte apoptosis plays a role in the activation of NAFLD/NASH. Apoptotic hepatocytes stimulate immune cells and hepatic stellate cells toward the progression of fibrosis in the liver through the production of inflammasomes and cytokines. Abnormalities in glucose and lipid metabolism as well as microbiota accelerate these processes. The production of reactive oxygen species, oxidative stress, and endoplasmic reticulum stress is also involved. Cell death, including apoptosis, seems very important in the progression of NAFLD and NASH. Recently, inhibitors of apoptosis have been developed as drugs for the treatment of NASH and may prevent cirrhosis and HCC. Increased hepatocyte apoptosis may distinguish NASH from

NAFLD, and the improvement of apoptosis could play a role in controlling the development of NASH. In this review, the association between apoptosis and NAFLD/NASH are discussed. This review could provide their knowledge, which plays a role in seeing the patients with NAFLD/NASH in daily clinical practice.

Core tip: Nonalcoholic fatty liver diseases (NAFLD), including nonalcoholic steatohepatitis (NASH), are one of the most serious health issues. We searched articles written in English and listed on PubMed for the role of apoptosis in NASH. There are close association between apoptosis and NAFLD/NASH. Several inhibitors of apoptosis have been suggested as potential treatments for NASH, and some are now being tested in clinical trials. Therefore, we should focus on the role of apoptosis in the progression of NAFLD/NASH.

INTRODUCTION

The term nonalcoholic fatty liver disease (NAFLD) includes nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). The mechanism by which NASH progresses is still largely unknown. Liver biopsy is an important procedure for the diagnosis of NASH, with a typical case of NASH having hepatocellular steatosis and ballooning, mixed acute and chronic lobular inflammation, and zone 3 perisinusoidal and pericellular fibrosis[1]. These findings have also been observed in the liver of patients with alcoholic steatohepatitis. NAFLD cirrhosis and NAFLD-hepatocellular carcinoma (HCC) are the second leading cause of liver transplants in the USA[2]. Accordingly, there is increasing evidence that HCC can develop in the NASH[2].

In the liver of patients with alcoholic hepatitis, infiltrating polymorphonuclear leukocytes and apoptotic bodies derived from hepatocytes are observed[3]. A combination of environmental factors, host genetics, and gut microbiota can lead to an excess accumulation of fat in the hepatocytes, which can result in lipotoxicity and trigger hepatocyte cell death, liver inflammation, fibrosis, and pathological angiogenesis, resulting in NASH, cirrhosis, and HCC[4]. In this topical review, we will discuss apoptosis, which is involved in the development of NASH.

APOPTOSIS AND THE ACTIVATION OF CASPASES IN THE PROGRESSION OF NASH

Feldstein *et al*[5] observed that terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive hepatocytes were significantly increased in the livers of NASH patients, compared to those from patients with alcoholic hepatitis or simple steatosis. Caspases have apoptotic functions as well as non-apoptotic functions. During apoptosis, the caspase cascade shapes the immunogenic properties of apoptosis[6]. Feldstein *et al*[5] found that active caspases 3 and 7 as well as the strong expression of Fas receptors in NASH specimens were strongly correlated with hepatocyte apoptosis and the progression of NASH. Caspase 3 activation and hepatocyte apoptosis are prominent features of different experimental models of NAFLD as well as human NAFLD and have been shown to be correlated with disease severity[5].

Caspase 3 is known to cleave several cellular substrates including cytokeratin-18 (CK-18), which is the major intermediate fragment protein in the liver[7]. Caspase 3 generated CK-18 fragments are an independent predictor of NASH in patients with suspected NAFLD[7,8]. Traffic-related air pollution has been shown to be associated with CK-18, a marker of hepatocellular apoptosis, in an overweight and obese pediatric population[9]. Mallory-Denk bodies (MDBs) are characteristic of both alcoholic and NASH and discriminate between the relatively benign simple steatosis and the more aggressive NASH. It has been shown that in genetically susceptible mice overexpressing CK-8, consumption of a high-fat diet (HFD) triggered hepatocellular injury, ballooning, apoptosis, inflammation, and MDB development[10].

Inhibition of hepatic apoptosis by pharmacological pan-caspase inhibitor VX-166 may reduce the development of fibrosis in mice with NASH[11,12]. Increases in active caspase 2, active caspase 3, and apoptosis were observed in the livers of patients with NASH[13]. Ballooned hepatocytes in NASH downregulate caspase 9, a pivotal caspase that executes the mitochondrial apoptosis pathway[14]. In rodents, a lack of caspase 8 expression in hepatocytes was shown to reduce the methionine-choline-deficient (MCD)-dependent increases in apoptosis, decreased the expression of pro-inflammatory cytokines, and reduced hepatic infiltration[15]. Caspase 8 may thus be critical for the pathogenesis of NASH.

Caspase 2 is an initiator caspase in lipid-induced cytotoxicity (lipoapoptosis), which plays a role in the pathogenesis of NASH[16]. Caspase 2 plays a role in lipid-induced hepatocyte apoptosis and is related to the production of apoptosis-associated fibrogenic factors[17]. Additionally, liver free coenzyme A content was shown to be reduced in mice with NASH. Decreased hepatic free coenzyme A content was associated with increased

caspase 2 activity and correlated with more severe liver cell apoptosis, inflammation, and fibrosis[18].

It has been reported that Fas, Fas ligand (FasL), and caspase 8 mRNA activation are important contributing factors to NAFLD[19]. Another study showed that children with NASH had significantly higher levels of soluble Fas and soluble FasL than those in the "not NASH" group[20]. Fas apoptosis inhibitory molecule (FAIM), a ubiquitously expressed antiapoptotic protein, functions as a mediator of Akt signaling[21]. Loss of FAIM leads to spontaneous obesity and hepatic steatosis[21].

Hepatic cell apoptosis is associated with miR-34a/Sirtuin 1 (SIRT1)/p53 signaling in NASH[22]. p53 and its transcriptional target, miR34a, have been shown to be involved in the pathogenesis of fatty liver. The p53 inhibitor, pifithrin-α-p-nitro, was shown to attenuate steatosis, associated oxidative stress, and apoptosis in murine models of NAFLD[23]. The DNA damage checkpoint protein Ataxia telangiectasia mutated pathway plays a role in the response to hepatic fat accumulation and promotes hepatocellular apoptosis and fibrosis in mice models of NAFLD[24]. Massive hepatic progenitor cell expansion, especially in children with NASH, is associated with the degree of liver injury, hepatocyte apoptosis, and cell-cycle arrest[25].

Increased vimentin fragment levels are known to indicate the existence of substantial hepatocellular apoptosis in the progression of NASH[26]. Levels of the augmenter of liver regeneration (ALR) protein were lower in liver tissues from patients with advanced alcoholic liver disease and nonalcoholic steatohepatitis than in liver tissues from controls[27]. Levels of steatosis and apoptosis were reduced in mice with a liver-specific deletion of ALR[27]. Impairment of the formation of a newly discovered ubiquitin ligase complex called linear ubiquitin chain assembly complex, has been shown to result in insufficient NF-κB activation and may thus be one of the molecular mechanisms underlying the enhanced apoptotic response of hepatocytes in NASH mouse models[28].

IgM-free apoptosis inhibitor of macrophage serum levels appear to be a sensitive diagnostic marker for NASH-HCC[29]. Activation of apoptosis signal-regulating kinase 1 (ASK1) in hepatocytes is a key step in the progression of nonalcoholic steatohepatitis[30]. Additionally, tumor necrosis factor alpha-induced protein 3 directly interacts with and deubiquitinates ASK1 in hepatocytes[30].

Thus, the activation of caspases and other molecules that are involved in apoptosis are frequently observed in the livers of NASH patients and may be related to the progression of NAFLD and NASH.

BCL-2 FAMILY MEMBERS AND MITOCHONDRIA IN THE PROGRESSION OF NASH

Liver injury in NASH patients is associated with apoptosis and NF-κB activation even though anti-apoptotic B-cell lymphoma 2 (Bcl-2) is strongly expressed[31,32]. These changes are caspase-dependent. They are also associated with mitochondrial membrane depolarization and the release of cytochrome c, which activate the mitochondrial apoptosis pathways including activation of the proteins Bcl-2-associated X (Bax) and Bcl-2-interacting mediator of cell death (Bim)[33]. The upregulation of Bax and Bcl-2 expression may also be play an important role in apoptosis in NAFLD[19], although it has been reported that NASH patients had significantly lower levels of anti-apoptotic protein Bcl-2[34]. The degree of apoptosis was inversely correlated with the level of Bcl-2[34].

Activation of endoplasmic reticulum (ER) stress-associated c-Jun N-terminal kinase (JNK) promotes apoptosis by modifying the expression and function of pro-apoptotic members of the Bcl-2 family such as Bcl-2 homology 3 (BH3) only protein Bim and p53-upregulated modulator of apoptosis (PUMA)[35]. PUMA promotes the activation of Bax and thus mitochondrial outer membrane permeabilization, which leads to the relocation of these pro-apoptotic mediators into cytosol[35]. Cazanave *et al*[36] reported that miR-296-5p levels were inversely related to the BH3-only protein PUMA mRNA levels in human liver specimens, and that miR-296-5p regulates PUMA expression during hepatic lipoapoptosis.

Transglutaminase 2 (TG2), which is induced in the nuclei of ethanol-treated hepatocytes, crosslinks and inactivates the transcription factor, SpI, which results in hepatic apoptosis[37]. In NASH patients, nuclear TG2 and crosslinked SpI formation were elevated. Additionally, activation of apoptosis inducing factor and a release of cytochrome c were observed[37]. Hypoxia, oxidative stress, and lipoapoptosis could all influence the expression of mitochondrial-encoded NADH dehydrogenase (MT-ND3) in hepatocytes and MT-ND3 may play a role in the progression of hepatic steatosis[38]. Hepatocyte-specific c-Met deletion in hepatocytes was shown to trigger NASH progression. Increased apoptosis was a prominent feature in c-MetΔ(hepa) livers[39]. Intermittent high glucose levels under lipotoxicity could contribute to the development of NAFLD by increasing oxidative stress and hepatocyte apoptosis via changes in mitochondrial permeability and subsequent mitochondrial dysfunction[40].

Bid promotes liver fibrosis coupled with a reduction of inflammation in experimental NASH models. In these models, hepatocyte apoptosis triggered hepatic stellate cell activation as well as liver fibrosis[41]. Increased expression of hepatocellular carcinoma down-regulated mitochondrial carrier protein (HDMCP) was identified in NASH animal models and HFFA-72h cultured L02 cells. The miR-146-HDMCP-downstream effector pathway is involved in NASH[42]. Collectively, previous studies have demonstrated that apoptosis resulting from mitochondrial injury is associated with the progression of NAFLD and NASH.

ACTIVATION OF JNK AND APOPTOSIS IN NASH

Monounsaturated and saturated fatty acids have been shown to induce cellular steatosis, apoptosis, and JNK activation in hepatocytes[33]. Steatotic hepatocytes from a murine NAFLD model were sensitive to TNF-α-induced apoptosis via the ASK1–JNK signaling pathway[43]. Free fatty acids (FFA)-induced ER stress is associated with JNK activation, which has been well documented in human steatosis[35]. Mixed lineage kinase 3 (MLK) 3 is one of the mitogen-activated protein kinases (MAP3K) that mediate JNK activation in the liver. MLK3 is involved in human NASH through JNK activation[44].

The interplay of p-JNK with mitochondrial Sab (Sh3bp5) leads to impaired respiration, production of reactive oxygen species (ROS), sustained JNK activation, apoptosis in condition of lipotoxicity, and ultimately contributes to the pathogenesis of NASH[45]. Dramatically reduced expression of cellular repressor of E1A-stimulated genes (CREG) and hyperactivated JNK1 signaling has been observed in the livers of NAFLD patients[46]. CREG is a robust suppressor of hepatic steatosis and metabolic disorders through its direct interaction with ASK1 and the subsequent inactivation of ASK1-JNK1 signaling[46]. Thus, JNK signaling pathways play an important role in the apoptosis of NAFLD and NASH.

APOPTOSIS, AND IMMUNE CELLS AND HEPATIC STELLATE CELLS

The complement cascade to clear apoptotic cells and promote liver regeneration is also involved in the progression of NAFLD and NASH[47]. Distant major histocompatibility complex class I-related chains A and B (MIC A/B) have been identified as ligands for the NK cell receptor G2D (NKG2D) in humans. Compared to controls, patients with NASH dysplayed increases in NKG2D and MIC A/B mRNA, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-death receptor 5 (DR5), CD95/Fas mRNA, and hepatocyte apoptosis[48]. These increases suggest that MIC A/B levels also affect the progression of NASH. TRAIL-producing natural killer (NK) cells actively promote a pro-inflammatory environment in the early stages of fatty liver disease, which suggests that this cell compartment may contribute to the progression of NASH[49].

Proliferation of hepatic macrophages, and the subsequent production of pro-inflammatory cytokines, initiate inflammatory cascades, orchestrate the activities of transcription factors involved in lipid metabolism/translocation, and modulate programmed cell death[50]. The macrophage activation marker-soluble CD163 was independently associated with the apoptosis marker CK-18 in Australian and Italian NAFLD patients[51]. Furthermore, down-modulation of NF-κB1 stimulates the progression of NASH in mice by promoting natural killer T (NKT)-cell-mediated responses[52]. Macrophage scavenger receptors), which play a role in the activation of signal transduction pathways that regulate inflammation, apoptotic cell clearance, chemoattraction and angiogenesis, are involved in both the early and advanced stages of NASH[53].

Wobser *et al*[54] has demonstrated that human hepatic stellate cells (HSCs) that were incubated with conditioned medium (CM) from steatotic hepatocytes and had fibrogenic activation and were resistant to apoptosis, which is important in the progression of fibrosis in chronic liver diseases[54]. Myeloperoxidase (MPO), a highly oxidative enzyme secreted by leukocytes, contributes to the activation of HSCs and is a part of a proapoptotic and profibrotic pathway of progression in NASH[55]. Thus, in patients with NAFLD and NASH, apoptotic hepatocytes stimulate immune cells and HSCs, which contributes to the progression of fibrosis in the liver.

PRO-INFLAMMATORY CYTOKINES AND CHEMOKINES

Feeding tumor necrosis factor (TNF) receptors 1 and 2 double-knock out mice (TNFRDKO mice) with an MCD-diet for 8 weeks attenuated liver steatosis and fibrosis and also suppressed hepatic induction of TNF-α, vascular cell adhesion molecule 1, and intracellular adhesion molecule 1, compared to wild-type control mice[6]. These results suggest that blocking the signaling of TNF receptors 1 and 2 is a promising therapeutic target for patients with NASH[56]. The TNF receptor 1-signaling pathway plays a role in aggravating a state of "simple steatosis" towards a phenotype with "NASH"[57]. Cyclooxygenase (COX)-2 may promote hepatocellular apoptosis by interacting with TNF-α and IL6 in rats with NASH[58]. COX-2 is highly expressed in NASH.

Lipoapoptotic supernatants stimulated monocyte migration to a similar magnitude as monocyte chemoattractant protein, CCL2 (MCP-1)[59]. The release of pannexin1-dependent pathophysiological eATP in lipoapoptosis can stimulate the migration of human monocytes in NASH[59]. In cultured Kupffer cells, cholesterol induced the expression of chemotactic and inflammatory cytokines (CCL2 and CXCL2, and IL1β, TNF and oncostatin M, respectively) and rendered hepatocytes more susceptible to apoptosis[60]. Lipids, which stimulate DR5, have been shown to induce the release of hepatocyte extracellular vesicles, which contain TRAILs. Lipids also induced the expression of IL1β and IL6 messenger RNAs in bone marrow-derived macrophages in mice[61]. The C-X-C motif chemokine 10 (CXCL10), which is known to be a pro-inflammation chemokine, was recently shown to play a pivotal role in the pathogenesis of NASH. By binding to its specific receptor CXCR3, CXCL10 recruits activated CXCR3+ T lymphocytes and macrophages to the parenchyma and promotes inflammation, apoptosis, and fibrosis[62].

The dsRNA receptor Nod-like receptor X1 and NLRP3

inflammasomes may be important in the development of NASH[63]. The function of receptor interacting protein kinase-3 (RIP3)-dependent "necroptosis" in NASH and NASH-induced fibrosis is currently unknown[64]. RIP3-dependent necroptosis controls NASH-induced liver fibrosis[64]. The absence of RIP3, a key mediator of necroptosis, exacerbates HFD-induced liver injury. This exacerbation is associated with increased inflammation and hepatocyte apoptosis as well as early fibrotic responses. These findings indicate that shifts in the mode of hepatocellular death can influence disease progression. Therefore, they may have therapeutic implications because manipulation of hepatocyte cell death pathways is currently considered to be a target for treatment of nonalcoholic fatty liver disease[65]. Thus, inflammasomes and cytokines induce apoptosis and respond to hepatocyte apoptosis in NAFLD and NASH.

OXIDATIVE STRESS

It has also been reported that oxidized phosphatidylcholine is localized in apoptotic hepatocytes in the livers of patients with the steatotic disorders, which indicates that oxidized phosphatidylcholine is formed in oxidatively damaged hepatocytes[66]. Transforming growth factor β (TGFβ) may regulate p53/p66Shc signaling in both the progression of human NASH and ROS levels and apoptosis[67]. NAFLD patients with reticuloendothelial system (RES) iron have increased TUNEL staining and cellular oxidative stress[68]. RES iron has been shown to be associated with NASH as well as more-severe histologic features[68].

TGFβ signaling activates Smad- and TGFβ-activated kinase 1-dependent signaling and plays a role in regulating cell survival, proliferation, fibrosis, and tumorigenesis. In hepatocytes, TGFβ signaling contributes to hepatocyte death and lipid accumulation through Smad signaling and ROS production, leading to the development of NASH[69]. NOX isoforms, including NOX1, NOX2 and NOX4, and NOX-derived ROS have all been implicated in regulating HSC activation and hepatocyte apoptosis. Both HSC activation and hepatocyte apoptosis are essential steps for the initiation of liver fibrosis and its progression[70]. Mainstream cigarette smoke has been shown to be associated with the degree of oxidative stress and hepatocellular apoptosis in NASH mice[71].

Oxidative stress is central to the pathogenesis of NASH. ROS are characterized by oxidative stress. ROS are generated in several cellular sites and their production is influenced by multi-organ interactions. For fatty liver diseases, mitochondrial dysfunction is the main source of ROS and is closely related to endoplasmic reticulum stress. Both are caused by lipotoxicity and together these three factors form a cycle of progressive organelle damage that results in sterile inflammation and apoptosis[72].

ER STRESS

FFAs can increase ER stress, leading to nuclear NF-κB activation and TG2 induction through the pancreatic ER kinase (PERK)-dependent pathways[37,73]. CCAAT/enhancer-binding protein homologous protein (CHOP) deficiency has been found to attenuate apoptosis, inflammation, fibrosis, and tumorigenesis in mice who are exposed to fat-loading conditions. This finding indicates CHOP promotes hepatocarcinogenesis in NASH[74]. The overexpression of hypoxia-inducible factor 1α (HIF-1α) has also been shown to blunt upregulation of the ER stress markers, CHOP and chaperone immunoglobulin heavy chain binding protein (GRP78/Bip), while knocking down HIF-1α increases the level of CHOP. These finding indicate that hepatocyte lipotoxicity is associated with decreased HIF-1α expression[75].

MiR-615-3p regulates lipoapoptosis by inhibiting CHOP and may be associated with the pathogenesis of NASH[76]. After exposure to saturated FFA, CHOP has been shown to induce hepatocyte cell apoptosis and inflammatory responses by activating NF-κB through a pathway involving the expression of IL1 receptor associated kinase 2. This activation results in the direct secretion of the cytokines IL8 and TNFα from hepatocytes[77]. Glucagon-like peptide-1 was found to protect against NAFLD by inactivating the ER stress-associated apoptosis pathway[78].

Loss of the unfolded protein response of regulator X-box binding protein 1 enhances injury in both *in vivo* and *in vitro* models of fatty liver injury[79]. Hepatocytes in a lipotoxic state ultimately undergo apoptosis through the upregulation of proteins involved in various pathways including PERK, CHOP, JNK, BIM, PUMA, and eventually, caspases[80].

AUTOPHAGY

Expression of microtubule associated protein 1 light chain 3α (LC3)-II, a hallmark of autophagic flux, was founded to be markedly increased in liver specimens from patients with NASH. JNK1 promotes palmitic acid-induced lipoapoptosis, whereas JNK2 activates pro-survival autophagy and inhibits palmitic acid lipotoxicity[81]. Palmitate may induce autophagy by activating the PKCα pathway in hepatocytes. Autophagy plays a protective role in palmitate-induced apoptosis in hepatocytes[82]. Tumor protein p53 binding protein 2 (ASPP2) is a pro-apoptotic member of the p53 binding protein family that inhibits autophagy[83]. Xie *et al*[83] reported that ASPP2 may participate in the lipid metabolism of non-alcoholic steatohepatitis. Mitochondrial uncoupling protein 2 (UCP2) also plays a role in the development of NASH[84]. Increasing UCP2 expression in hepatoma cells may contribute to cell autophagy and may inhibit apoptosis as result of fatty acid injury[84]. Cellular degradation of Kelch-like ECH-associated protein 1 through the progress of sequestrosome (SQSTM)1/p62-dependent autophagy

activates JNK, upregulates expression of Bim and PUMA, and contributes to hepatocyte apoptosis induced by saturated FFAs[85]. Parkin-mediated mitophagy may mitigate hepatocyte apoptosis, improve mitochondrial quality, and suppress steatosis (lipid accumulation) in animal models of alcoholic fatty liver disease[86]. In rats treated with ethanol-enhanced hepatic mitophagy was associated with Parkin mitochondrial translocation, which was triggered by oxidative mitochondrial DNA damage[86]. Rubicon is overexpressed and plays a pathogenic role in NAFLD by accelerating hepatocellular lipoapoptosis and lipid accumulation and inhibiting autophagy[87]. Sirtuin 3 (SIRT3) is a nicotinamide adenine dinucleotide-dependent deacetylase that is primarily located inside the mitochondria[88]. SIRT3 negatively regulates autophagy, thereby enhancing the susceptibility of hepatocytes to SFA-induced cytotoxicity[88].

Thus, ROS production, oxidative stress, and ER stress are all known to induce apoptosis. Autophagy modifies the progression of NAFLD and NASH and may have a protective role in hepatocyte apoptosis.

GLUCOSE METABOLISM AND APOPTOSIS

Hepatic insulin signaling is impaired in NASH patients, where downregulation of insulin-sensitive targets is associated with increased apoptosis and fibrogenesis[89]. Hyperinsulinemia has been shown to alter nuclear transcriptional regulators of cholesterol homeostasis. This leads hepatic accumulation of free cholesterol, hepatic injury, and apoptosis in NASH patients[90].

Fibroblast growth factor (FGF)-21 is highly expressed in the liver and regulates glucose and lipid metabolism in rodents. Concentration of FGF-21 were found to be significantly and independently correlated with hepatic fat content and markers of hepatic apoptosis in obese youth[91]. Another study found that FGF-21 mRNA expression in the human liver increased with steatosis grade and that its serum level is significantly elevated in adult NAFLD patients[92].

Intrahepatic expression of dipeptidyl peptidase-4 (DPP4) and circulating DPP4 (cDPP4) levels and its enzymatic activity are all increased in NAFLD[93]. Circulating DPP4 activity correlates with measures of hepatocyte apoptosis and fibrosis in NAFLD in patients with type 2 diabetes mellitus and/or obesity[93]. Senescence marker protein-30 is involved in both glucose metabolism disorder and NAFLD[94].

TRAIL receptor signaling was also found to be involved in the pathogenesis of NASH in mice with a genetic deletion of the TRAIL receptor[95]. Furthermore, patients with NASH had significantly reduced plasma TRAIL concentrations compared to controls, patients with simple steatosis, or obese individuals[96]. TRAIL protects against insulin resistance, NAFLD, and vascular inflammation. Increasing TRAIL levels may be an attractive therapeutic strategy for reducing symptoms

of diabetes as well as liver and vascular injuries, which are commonly observed in individuals with NAFLD[96].

LIPID METABOLISM AND APOPTOSIS

The serine/threonine kinases, glycogen synthase kinase GSK-3α and GSK-3β, can participate in pro-apoptotic signaling during FFA-induced lipoapoptosis[35]. More specifically, saturated fatty acids strongly induce hepatocyte apoptosis[97]. Saturated fatty acids up-regulate the inflammasome in hepatocytes and lead to sensitization to LPS-induced inflammasome activation and inflammatory injury[98,99]. Saturated fatty acids also induce hepatocyte apoptosis and the activation of caspase 8, which triggers the release of dangerous molecules[98].

Resistance to lipoapoptosis is, in part, due to an autocrine hedgehog signaling pathway[14]. Farnesoid X receptor is a member of the nuclear receptor superfamily that plays a crucial role in bile acid, cholesterol, lipid, and glucose metabolism as well as apoptosis[100]. It is also involved in the pathogenesis of NASH. The cellular inhibitor of apoptosis proteins 1 and 2 (cIAP-1 and cIAP-2) are potent inhibitors of death receptor-mediated apoptosis. Proteasomal degradation of cIAPs by FFA contributes to hepatocyte lipoapoptosis[101]. Palmitate-induced lipoapoptosis is dependent on calcium-stimulated mitochondrial activation, which induces oxidative stress and hepatic cell lipotoxicity[102].

Free cholesterol accumulates in NASH patients but not in simple steatosis. Mitochondrial free cholesterol deposition causes hepatocyte apoptosis and necrosis by activating JNK1[103]. High-mobility-group-box 1 and toll-like receptor 4 are both involved in this activation mechanism[103]. Cholesterol markedly promoted the apoptosis of steatosis HepG2 cells *in vitro*, likely through the up-regulation of expression of Bax and caspase 3[104]. Palmitate activation by fatty acid transport protein 4 triggers hepatocellular apoptosis via altered phospholipid composition and steatosis by acylation into complex lipids[105]. These complex lipids are involved in the development of NAFLD[105]. E2F transcription factors are known regulators of the cell cycle, proliferation, apoptosis, and differentiation. E2F1 regulates lipid synthesis and glycolysis and thus contributes to the development of NAFLD[106].

Androgen-dependent proapoptotic polycystic ovarian syndrome (PCOS) may directly contribute to NAFLD progression in PCOS patients[107]. A recent study gave a complementary fast food (FF) diet to a NASH mouse model, thus mimicking features of the metabolic syndrome. The study found that miR-21 levels increased in both the liver and muscle and expression of peroxisome proliferator-activated receptor α, a key miR-21 target, was decreased[108]. In a typical model of NASH-associated liver damage, miR-21 ablation results in a progressive decrease in steatosis, inflammation and lipoapoptosis, with a subsequent impairment of fibrosis[108].

Table 1 Inhibitors of apoptosis for nonalcoholic steatohepatitis

Drug and reagents	Targets	Mechanism of action	Ref.
Triacsin C	Intracellular long-chain acyl-CoA synthetases (ACSL)	Triacylglycerol (TAG) accumulation into lipid droplets	[112]
Ezetimibe	AMPK phosphorylation	TFEB-mediated activation of autophagy and NLRP3 inflammasome inhibition	[113]
Baicalin	Inhibition of NF-κB	NF-κB anti-inflammation signaling pathways	[114]
Granulocyte colony stimulating factor (G-CSF)	PI3K/Akt	Activation of PI3K and Akt pathway	[115]
Elafibranor (GFT505)	PPARα/δ	Agonist of PPARα/δ receptors	[116]
Isoquercitrin	Dipeptidyl peptidase-IV(DPP-IV)	Activation of glucagonlike peptide-1 (GLP-1)	[117]
Activated carbon N-acetylcysteine (ACNAC) microcapsules	Telomerase	Improved telomerase activity	[118]
3-Acetyl-oleanolic acid (3Ac-OA)	Glucose transporter type 2 (GLUT-2), low-density lipoprotein receptor (LDLR)	AMPK-related pathways	[119]
Meretrix oligopeptides (MMO)	NF-κB	NF-κB anti-inflammation signaling pathways	[120]
Seladelpar (MBX-8025)	Proliferator-activated receptor- delta (PPAR-δ)	Selective PPAR-δ agonist	[121]
Resveratrol	Sirt1	Antioxidant	[122]
TBE-31	NF-E2 p45-related factor 2 (Nrf2)	Regulation of intracellular redox homeostasis	[124]

Figure 1 Disease progression of nonalcoholic fatty liver diseases and nonalcoholic steatohepatitis. The exact role of autophagy in nonalcoholic fatty liver diseases/nonalcoholic steatohepatitis remains unclear. NAFLD: Nonalcoholic fatty liver diseases; NASH: Nonalcoholic steatohepatitis.

MICROBIOTA AND APOPTOSIS

Intestinal endotoxin [lipopolysaccharide (LPS)] augments liver injury in MCD mice[109]. In a recent study, a group of male C57BL/6 mice were fed with a MCD diet for 17 days, injected with LPS intraperitoneally, and sacrificed 6 h after LPS injection. The study found that LPS upregulated TNF-α production, which induce hepatocyte apoptosis[110]. Palmitate and lysophosphatidylcholine (LPC) induced upregulation of the p53-upregulated modulator of apoptosis and cell-surface expression of the death receptor TNF-related apoptosis-inducing ligand receptor 2[111]. In part, microbiota may be involved in the progression of NAFLD and NASH through hepatocyte apoptosis.

CONCLUSION

Cell death, including apoptosis, seems important in the

progression of NAFLD and NASH (Figure 1). Recently, several inhibitors of apoptosis have been suggested as potential treatments for NASH (Table 1)[112-124]. Clinical trials for the treatment of NASH are currently being conducted[125] and some are targeting apoptosis in NASH patients. Increased hepatocyte apoptosis may distinguish NASH from NAFLD[126]. Repair responses may play an important role in controlling the disease severities of NASH[126]. We reviewed published articles related to this topic and discussed the importance of apoptosis in NAFLD and NASH.

REFERENCES

1   **Brunt EM**, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol* 1999; **94**: 2467-2474 [PMID: 10484010 DOI: 10.1111/j.1572-0241.1999.01377.x]

2   **Bellentani S**. The epidemiology of non-alcoholic fatty liver

disease. *Liver Int* 2017; **37** Suppl 1: 81-84 [PMID: 28052624 DOI: 10.1111/liv.13299]

3   **Takahashi T**, So-Wan T, Kamimura T, Asakura H. Infiltrating polymorphonuclear leukocytes and apoptotic bodies derived from hepatocytes but not from ballooning hepatocytes containing Mallory bodies show nuclear DNA fragmentation in alcoholic hepatitis. *Alcohol Clin Exp Res* 2000; **24**: 68S-73S [PMID: 10803784]

4   **Povero D**, Feldstein AE. Novel Molecular Mechanisms in the Development of Non-Alcoholic Steatohepatitis. *Diabetes Metab J* 2016; **40**: 1-11 [PMID: 26912150 DOI: 10.4093/dmj.2016.40.1.1]

5   **Feldstein AE**, Canbay A, Angulo P, Taniai M, Burgart LJ, Lindor KD, Gores GJ. Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. *Gastroenterology* 2003; **125**: 437-443 [PMID: 12891546]

6   **McArthur K**, Kile BT. Apoptotic Caspases: Multiple or Mistaken Identities? *Trends Cell Biol* 2018; **28**: 475-493 [PMID: 29551258 DOI: 10.1016/j.tcb.2018.02.003]

7   **Wieckowska A**, Zein NN, Yerian LM, Lopez AR, McCullough AJ, Feldstein AE. In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. *Hepatology* 2006; **44**: 27-33 [PMID: 16799979]

8   **Feldstein AE**, Wieckowska A, Lopez AR, Liu YC, Zein NN, McCullough AJ. Cytokeratin-18 fragment levels as noninvasive biomarkers for nonalcoholic steatohepatitis: a multicenter validation study. *Hepatology* 2009; **50**: 1072-1078 [PMID: 19585618 DOI: 10.1002/hep.23050]

9   **Hsieh S**, Leaderer BP, Feldstein AE, Santoro N, McKay LA, Caprio S, McConnell R. Traffic-related air pollution associations with cytokeratin-18, a marker of hepatocellular apoptosis, in an overweight and obese paediatric population. *Pediatr Obes* 2018; **13**: 342-347 [PMID: 28730729 DOI: 10.1111/ijpo.12228]

10   **Kucukoglu O**, Guldiken N, Chen Y, Usachov V, El-Heliebi A, Haybaeck J, Denk H, Trautwein C, Strnad P. High-fat diet triggers Mallory-Denk body formation through misfolding and crosslinking of excess keratin 8. *Hepatology* 2014; **60**: 169-178 [PMID: 24519272 DOI: 10.1002/hep.27068]

11   **Witek RP**, Stone WC, Karaca FG, Syn WK, Pereira TA, Agboola KM, Omenetti A, Jung Y, Teaberry V, Choi SS, Guy CD, Pollard J, Charlton P, Diehl AM. Pan-caspase inhibitor VX-166 reduces fibrosis in an animal model of nonalcoholic steatohepatitis. *Hepatology* 2009; **50**: 1421-1430 [PMID: 19676126 DOI: 10.1002/hep.23167]

12   **Anstee QM**, Concas D, Kudo H, Levene A, Pollard J, Charlton P, Thomas HC, Thursz MR, Goldin RD. Impact of pan-caspase inhibition in animal models of established steatosis and non-alcoholic steatohepatitis. *J Hepatol* 2010; **53**: 542-550 [PMID: 20557969 DOI: 10.1016/j.jhep.2010.03.016]

13   **Ferreira DM**, Castro RE, Machado MV, Evangelista T, Silvestre A, Costa A, Coutinho J, Carepa F, Cortez-Pinto H, Rodrigues CM. Apoptosis and insulin resistance in liver and peripheral tissues of morbidly obese patients is associated with different stages of non-alcoholic fatty liver disease. *Diabetologia* 2011; **54**: 1788-1798 [PMID: 21455726 DOI: 10.1007/s00125-011-2130-8]

14   **Kakisaka K**, Cazanave SC, Werneburg NW, Razumilava N, Mertens JC, Bronk SF, Gores GJ. A hedgehog survival pathway in 'undead' lipotoxic hepatocytes. *J Hepatol* 2012; **57**: 844-851 [PMID: 22641094 DOI: 10.1016/j.jhep.2012.05.011]

15   **Hatting M**, Zhao G, Schumacher F, Sellge G, Al Masaoudi M, Gaßler N, Boekschoten M, Müller M, Liedtke C, Cubero FJ, Trautwein C. Hepatocyte caspase-8 is an essential modulator of steatohepatitis in rodents. *Hepatology* 2013; **57**: 2189-2201 [PMID: 23339067 DOI: 10.1002/hep.26271]

16   **Johnson ES**, Lindblom KR, Robeson A, Stevens RD, Ilkayeva OR, Newgard CB, Kornbluth S, Andersen JL. Metabolomic profiling reveals a role for caspase-2 in lipoapoptosis. *J Biol Chem* 2013; **288**: 14463-14475 [PMID: 23553630 DOI: 10.1074/jbc.M112.437210]

17   **Machado MV**, Michelotti GA, Pereira Tde A, Boursier J, Kruger L, Swiderska-Syn M, Karaca G, Xie G, Guy CD, Bohinc B, Lindblom

KR, Johnson E, Kornbluth S, Diehl AM. Reduced lipoapoptosis, hedgehog pathway activation and fibrosis in caspase-2 deficient mice with non-alcoholic steatohepatitis. *Gut* 2015; **64**: 1148-1157 [PMID: 25053716 DOI: 10.1136/gutjnl-2014-307362]

18   **Machado MV**, Kruger L, Jewell ML, Michelotti GA, Pereira Tde A, Xie G, Moylan CA, Diehl AM. Vitamin B5 and N-Acetylcysteine in Nonalcoholic Steatohepatitis: A Preclinical Study in a Dietary Mouse Model. *Dig Dis Sci* 2016; **61**: 137-148 [PMID: 26403427 DOI: 10.1007/s10620-015-3871-x]

19   **Li CP**, Li JH, He SY, Li P, Zhong XL. Roles of Fas/Fasl, Bcl-2/Bax, and Caspase-8 in rat nonalcoholic fatty liver disease pathogenesis. *Genet Mol Res* 2014; **13**: 3991-3999 [PMID: 24938610 DOI: 10.4238/2014.May.23.10]

20   **Alkhouri N**, Alisi A, Okwu V, Matloob A, Ferrari F, Crudele A, De Vito R, Lopez R, Feldstein AE, Nobili V. Circulating Soluble Fas and Fas Ligand Levels Are Elevated in Children with Nonalcoholic Steatohepatitis. *Dig Dis Sci* 2015; **60**: 2353-2359 [PMID: 25764498 DOI: 10.1007/s10620-015-3614-z]

21   **Huo J**, Ma Y, Liu JJ, Ho YS, Liu S, Soh LY, Chen S, Xu S, Han W, Hong A, Lim SC, Lam KP. Loss of Fas apoptosis inhibitory molecule leads to spontaneous obesity and hepatosteatosis. *Cell Death Dis* 2016; **7**: e2091 [PMID: 26866272 DOI: 10.1038/cddis.2016.12]

22   **Castro RE**, Ferreira DM, Afonso MB, Borralho PM, Machado MV, Cortez-Pinto H, Rodrigues CM. miR-34a/SIRT1/p53 is suppressed by ursodeoxycholic acid in the rat liver and activated by disease severity in human non-alcoholic fatty liver disease. *J Hepatol* 2013; **58**: 119-125 [PMID: 22902550 DOI: 10.1016/j.jhep.2012.08.008]

23   **Derdak Z**, Villegas KA, Harb R, Wu AM, Sousa A, Wands JR. Inhibition of p53 attenuates steatosis and liver injury in a mouse model of non-alcoholic fatty liver disease. *J Hepatol* 2013; **58**: 785-791 [PMID: 23211317 DOI: 10.1016/j.jhep.2012.11.042]

24   **Daugherity EK**, Balmus G, Al Saei A, Moore ES, Abi Abdallah D, Rogers AB, Weiss RS, Maurer KJ. The DNA damage checkpoint protein ATM promotes hepatocellular apoptosis and fibrosis in a mouse model of non-alcoholic fatty liver disease. *Cell Cycle* 2012; **11**: 1918-1928 [PMID: 22544329 DOI: 10.4161/cc.20259]

25   **Nobili V**, Carpino G, Alisi A, Franchitto A, Alpini G, De Vito R, Onori P, Alvaro D, Gaudio E. Hepatic progenitor cells activation, fibrosis, and adipokines production in pediatric nonalcoholic fatty liver disease. *Hepatology* 2012; **56**: 2142-2153 [PMID: 22467277 DOI: 10.1002/hep.25742]

26   **Lee SJ**, Yoo JD, Choi SY, Kwon OS. The expression and secretion of vimentin in the progression of non-alcoholic steatohepatitis. *BMB Rep* 2014; **47**: 457-462 [PMID: 24325816]

27   **Gandhi CR**, Chaillet JR, Nalesnik MA, Kumar S, Dangi A, Demetris AJ, Ferrell R, Wu T, Divanovic S, Stankeiwicz T, Shaffer B, Stolz DB, Harvey SA, Wang J, Starzl TE. Liver-specific deletion of augmenter of liver regeneration accelerates development of steatohepatitis and hepatocellular carcinoma in mice. *Gastroenterology* 2015; **148**: 379-391.e4 [PMID: 25448926 DOI: 10.1053/j.gastro.2014.10.008]

28   **Matsunaga Y**, Nakatsu Y, Fukushima T, Okubo H, Iwashita M, Sakoda H, Fujishiro M, Yamamotoya T, Kushiyama A, Takahashi S, Tsuchiya Y, Kamata H, Tokunaga F, Iwai K, Asano T. LUBAC Formation Is Impaired in the Livers of Mice with MCD-Dependent Nonalcoholic Steatohepatitis. *Mediators Inflamm* 2015; **2015**: 125380 [PMID: 26170532 DOI: 10.1155/2015/125380]

29   **Koyama N**, Yamazaki T, Kanetsuki Y, Hirota J, Asai T, Mitsumoto Y, Mizuno M, Shima T, Kanbara Y, Arai S, Miyazaki T, Okanoue T. Activation of apoptosis inhibitor of macrophage is a sensitive diagnostic marker for NASH-associated hepatocellular carcinoma. *J Gastroenterol* 2018; **53**: 770-779 [PMID: 29086016 DOI: 10.1007/s00535-017-1398-y]

30   **Zhang P**, Wang PX, Zhao LP, Zhang X, Ji YX, Zhang XJ, Fang C, Lu YX, Yang X, Gao MM, Zhang Y, Tian S, Zhu XY, Gong J, Ma XL, Li F, Wang Z, Huang Z, She ZG, Li H. The deubiquitinating enzyme TNFAIP3 mediates inactivation of hepatic ASK1 and ameliorates nonalcoholic steatohepatitis. *Nat Med* 2018; **24**: 84-94

[PMID: 29227477 DOI: 10.1038/nm.4453]

31   **Ramalho RM**, Cortez-Pinto H, Castro RE, Solá S, Costa A, Moura MC, Camilo ME, Rodrigues CM. Apoptosis and Bcl-2 expression in the livers of patients with steatohepatitis. *Eur J Gastroenterol Hepatol* 2006; **18**: 21-29 [PMID: 16357615]

32   **Ribeiro PS**, Cortez-Pinto H, Solá S, Castro RE, Ramalho RM, Baptista A, Moura MC, Camilo ME, Rodrigues CM. Hepatocyte apoptosis, expression of death receptors, and activation of NF-kappaB in the liver of nonalcoholic and alcoholic steatohepatitis patients. *Am J Gastroenterol* 2004; **99**: 1708-1717 [PMID: 15330907]

33   **Malhi H**, Bronk SF, Werneburg NW, Gores GJ. Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis. *J Biol Chem* 2006; **281**: 12093-12101 [PMID: 16505490]

34   **El Bassat H**, Ziada DH, Hasby EA, Nagy H, Abo Ryia MH. Apoptotic and anti-apoptotic seromarkers for assessment of disease severity of non-alcoholic steatohepatitis. *Arab J Gastroenterol* 2014; **15**: 6-11 [PMID: 24630506 DOI: 10.1016/j.ajg.2014.01.009]

35   **Ibrahim SH**, Akazawa Y, Cazanave SC, Bronk SF, Elmi NA, Werneburg NW, Billadeau DD, Gores GJ. Glycogen synthase kinase-3 (GSK-3) inhibition attenuates hepatocyte lipoapoptosis. *J Hepatol* 2011; **54**: 765-772 [PMID: 21147505 DOI: 10.1016/j.jhep.2010.09.039]

36   **Cazanave SC**, Mott JL, Elmi NA, Bronk SF, Masuoka HC, Charlton MR, Gores GJ. A role for miR-296 in the regulation of lipoapoptosis by targeting PUMA. *J Lipid Res* 2011; **52**: 1517-1525 [PMID: 21633093 DOI: 10.1194/jlr.M014654]

37   **Kuo TF**, Tatsukawa H, Matsuura T, Nagatsuma K, Hirose S, Kojima S. Free fatty acids induce transglutaminase 2-dependent apoptosis in hepatocytes via ER stress-stimulated PERK pathways. *J Cell Physiol* 2012; **227**: 1130-1137 [PMID: 21567402 DOI: 10.1002/jcp.22833]

38   **Wang HN**, Chen HD, Chen KY, Xiao JF, He K, Xiang GA, Xie X. Highly expressed MT-ND3 positively associated with histological severity of hepatic steatosis. *APMIS* 2014; **122**: 443-451 [PMID: 24020820 DOI: 10.1111/apm.12166]

39   **Kroy DC**, Schumacher F, Ramadori P, Hatting M, Bergheim I, Gassler N, Boekschoten MV, Müller M, Streetz KL, Trautwein C. Hepatocyte specific deletion of c-Met leads to the development of severe non-alcoholic steatohepatitis in mice. *J Hepatol* 2014; **61**: 883-890 [PMID: 24845607 DOI: 10.1016/j.jhep.2014.05.019]

40   **Yin X**, Zheng F, Pan Q, Zhang S, Yu D, Xu Z, Li H. Glucose fluctuation increased hepatocyte apoptosis under lipotoxicity and the involvement of mitochondrial permeability transition opening. *J Mol Endocrinol* 2015; **55**: 169-181 [PMID: 26464382 DOI: 10.1530/JME-15-0101]

41   **Eguchi A**, De Mollerat Du Jeu X, Johnson CD, Nektaria A, Feldstein AE. Liver Bid suppression for treatment of fibrosis associated with non-alcoholic steatohepatitis. *J Hepatol* 2016; **64**: 699-707 [PMID: 26555271 DOI: 10.1016/j.jhep.2015.11.002]

42   **Jin X**, Liu J, Chen YP, Xiang Z, Ding JX, Li YM. Effect of miR-146 targeted HDMCP up-regulation in the pathogenesis of nonalcoholic steatohepatitis. *PLoS One* 2017; **12**: e0174218 [PMID: 28346483 DOI: 10.1371/journal.pone.0174218]

43   **Zhang W**, Kudo H, Kawai K, Fujisaka S, Usui I, Sugiyama T, Tsukada K, Chen N, Takahara T. Tumor necrosis factor-alpha accelerates apoptosis of steatotic hepatocytes from a murine model of non-alcoholic fatty liver disease. *Biochem Biophys Res Commun* 2010; **391**: 1731-1736 [PMID: 20043871 DOI: 10.1016/j.bbrc.2009.12.144]

44   **Ibrahim SH**, Gores GJ, Hirsova P, Kirby M, Miles L, Jaeschke A, Kohli R. Mixed lineage kinase 3 deficient mice are protected against the high fat high carbohydrate diet-induced steatohepatitis. *Liver Int* 2014; **34**: 427-437 [PMID: 24256559 DOI: 10.1111/liv.12353]

45   **Win S**, Than TA, Le BH, García-Ruiz C, Fernandez-Checa JC, Kaplowitz N. Sab (Sh3bp5) dependence of JNK mediated inhibition of mitochondrial respiration in palmitic acid induced hepatocyte lipotoxicity. *J Hepatol* 2015; **62**: 1367-1374 [PMID: 25666017 DOI: 10.1016/j.jhep.2015.01.032]

46   **Zhang QY**, Zhao LP, Tian XX, Yan CH, Li Y, Liu YX, Wang PX, Zhang XJ, Han YL. The novel intracellular protein CREG inhibits hepatic steatosis, obesity, and insulin resistance. *Hepatology* 2017; **66**: 834-854 [PMID: 28508477 DOI: 10.1002/hep.29257]

47   **Rensen SS**, Slaats Y, Driessen A, Peutz-Kootstra CJ, Nijhuis J, Steffensen R, Greve JW, Buurman WA. Activation of the complement system in human nonalcoholic fatty liver disease. *Hepatology* 2009; **50**: 1809-1817 [PMID: 19821522 DOI: 10.1002/hep.23228]

48   **Kahraman A**, Schlattjan M, Kocabayoglu P, Yildiz-Meziletoglu S, Schlensak M, Fingas CD, Wedemeyer I, Marquitan G, Gieseler RK, Baba HA, Gerken G, Canbay A. Major histocompatibility complex class I-related chains A and B (MIC A/B): a novel role in nonalcoholic steatohepatitis. *Hepatology* 2010; **51**: 92-102 [PMID: 19998387 DOI: 10.1002/hep.23253]

49   **Gomez-Santos L**, Luka Z, Wagner C, Fernandez-Alvarez S, Lu SC, Mato JM, Martinez-Chantar ML, Beraza N. Inhibition of natural killer cells protects the liver against acute injury in the absence of glycine N-methyltransferase. *Hepatology* 2012; **56**: 747-759 [PMID: 22392635 DOI: 10.1002/hep.25694]

50   **Wu R**, Nakatsu G, Zhang X, Yu J. Pathophysiological mechanisms and therapeutic potentials of macrophages in non-alcoholic steatohepatitis. *Expert Opin Ther Targets* 2016; **20**: 615-626 [PMID: 26609894 DOI: 10.1517/14728222.2016.1125883]

51   **Kazankov K**, Barrera F, Møller HJ, Rosso C, Bugianesi E, David E, Ibrahim Kamal Jouness R, Esmaili S, Eslam M, McLeod D, Bibby BM, Vilstrup H, George J, Grønbaek H. The macrophage activation marker sCD163 is associated with morphological disease stages in patients with non-alcoholic fatty liver disease. *Liver Int* 2016; **36**: 1549-1557 [PMID: 27102725 DOI: 10.1111/liv.13150]

52   **Locatelli I**, Sutti S, Vacchiano M, Bozzola C, Albano E. NF-κB1 deficiency stimulates the progression of non-alcoholic steatohepatitis (NASH) in mice by promoting NKT-cell-mediated responses. *Clin Sci* (Lond) 2013; **124**: 279-287 [PMID: 22970906 DOI: 10.1042/CS20120289]

53   **Bieghs V**, Wouters K, van Gorp PJ, Gijbels MJ, de Winther MP, Binder CJ, Lütjohann D, Febbraio M, Moore KJ, van Bilsen M, Hofker MH, Shiri-Sverdlov R. Role of scavenger receptor A and CD36 in diet-induced nonalcoholic steatohepatitis in hyperlipidemic mice. *Gastroenterology* 2010; **138**: 2477-2486, 2486.e1-2486.e3 [PMID: 20206177 DOI: 10.1053/j.gastro.2010.02.051]

54   **Wobser H**, Dorn C, Weiss TS, Amann T, Bollheimer C, Büttner R, Schölmerich J, Hellerbrand C. Lipid accumulation in hepatocytes induces fibrogenic activation of hepatic stellate cells. *Cell Res* 2009; **19**: 996-1005 [PMID: 19546889 DOI: 10.1038/cr.2009.73]

55   **Pulli B**, Ali M, Iwamoto Y, Zeller MW, Schob S, Linnoila JJ, Chen JW. Myeloperoxidase-Hepatocyte-Stellate Cell Cross Talk Promotes Hepatocyte Injury and Fibrosis in Experimental Nonalcoholic Steatohepatitis. *Antioxid Redox Signal* 2015; **23**: 1255-1269 [PMID: 26058518 DOI: 10.1089/ars.2014.6108]

56   **Tomita K**, Tamiya G, Ando S, Ohsumi K, Chiyo T, Mizutani A, Kitamura N, Toda K, Kaneko T, Horie Y, Han JY, Kato S, Shimoda M, Oike Y, Tomizawa M, Makino S, Ohkura T, Saito H, Kumagai N, Nagata H, Ishii H, Hibi T. Tumour necrosis factor alpha signalling through activation of Kupffer cells plays an essential role in liver fibrosis of non-alcoholic steatohepatitis in mice. *Gut* 2006; **55**: 415-424 [PMID: 16174657]

57   **Aparicio-Vergara M**, Hommelberg PP, Schreurs M, Gruben N, Stienstra R, Shiri-Sverdlov R, Kloosterhuis NJ, de Bruin A, van de Sluis B, Koonen DP, Hofker MH. Tumor necrosis factor receptor 1 gain-of-function mutation aggravates nonalcoholic fatty liver disease but does not cause insulin resistance in a murine model. *Hepatology* 2013; **57**: 566-576 [PMID: 22941955 DOI: 10.1002/hep.26046]

58   **Cheng Q**, Li N, Chen M, Zheng J, Qian Z, Wang X, Huang C, Xu S, Shi G. Cyclooxygenase-2 promotes hepatocellular apoptosis by interacting with TNF-α and IL-6 in the pathogenesis of nonalcoholic steatohepatitis in rats. *Dig Dis Sci* 2013; **58**: 2895-2902 [PMID: 23975340 DOI: 10.1007/s10620-013-2823-6]

59   **Xiao F**, Waldrop SL, Bronk SF, Gores GJ, Davis LS, Kilic G. Lipoapoptosis induced by saturated free fatty acids stimulates monocyte migration: a novel role for Pannexin1 in liver cells. *Purinergic Signal* 2015; **11**: 347-359 [PMID: 26054298 DOI: 10.1007/s11302-015-9456-5]

60   **Henkel J**, Coleman CD, Schraplau A, Johrens K, Weber D, Castro JP, Hugo M, Schulz TJ, Krämer S, Schürmann A, Püschel GP. Induction of steatohepatitis (NASH) with insulin resistance in wildtype B6 mice by a western-type diet containing soybean oil and cholesterol. *Mol Med* 2017; **23**: Epub ahead of print [PMID: 28332698 DOI: 10.2119/molmed.2016.00203]

61   **Hirsova P**, Ibrahim SH, Krishnan A, Verma VK, Bronk SF, Werneburg NW, Charlton MR, Shah VH, Malhi H, Gores GJ. Lipid-Induced Signaling Causes Release of Inflammatory Extracellular Vesicles From Hepatocytes. *Gastroenterology* 2016; **150**: 956-967 [PMID: 26764184 DOI: 10.1053/j.gastro.2015.12.037]

62   **Xu Z**, Zhang X, Lau J, Yu J. C-X-C motif chemokine 10 in non-alcoholic steatohepatitis: role as a pro-inflammatory factor and clinical implication. *Expert Rev Mol Med* 2016; **18**: e16 [PMID: 27669973]

63   **Wang YG**, Fang WL, Wei J, Wang T, Wang N, Ma JL, Shi M. The involvement of NLRX1 and NLRP3 in the development of nonalcoholic steatohepatitis in mice. *J Chin Med Assoc* 2013; **76**: 686-692 [PMID: 24084392 DOI: 10.1016/j.jcma.2013.08.010]

64   **Gautheron J**, Vucur M, Reisinger F, Cardenas DV, Roderburg C, Koppe C, Kreggenwinkel K, Schneider AT, Bartneck M, Neumann UP, Canbay A, Reeves HL, Luedde M, Tacke F, Trautwein C, Heikenwalder M, Luedde T. A positive feedback loop between RIP3 and JNK controls non-alcoholic steatohepatitis. *EMBO Mol Med* 2014; **6**: 1062-1074 [PMID: 24963148 DOI: 10.15252/emmm.201403856]

65   **Roychowdhury S**, McCullough RL, Sanz-Garcia C, Saikia P, Alkhouri N, Matloob A, Pollard KA, McMullen MR, Croniger CM, Nagy LE. Receptor interacting protein 3 protects mice from high-fat diet-induced liver injury. *Hepatology* 2016; **64**: 1518-1533 [PMID: 27301788 DOI: 10.1002/hep.28676]

66   **Ikura Y**, Ohsawa M, Suekane T, Fukushima H, Itabe H, Jomura H, Nishiguchi S, Inoue T, Naruko T, Ehara S, Kawada N, Arakawa T, Ueda M. Localization of oxidized phosphatidylcholine in nonalcoholic fatty liver disease: impact on disease progression. *Hepatology* 2006; **43**: 506-514 [PMID: 16496325]

67   **Tomita K**, Teratani T, Suzuki T, Oshikawa T, Yokoyama H, Shimamura K, Nishiyama K, Mataki N, Irie R, Minamino T, Okada Y, Kurihara C, Ebinuma H, Saito H, Shimizu I, Yoshida Y, Hokari R, Sugiyama K, Hatsuse K, Yamamoto J, Kanai T, Miura S, Hibi T. p53/p66Shc-mediated signaling contributes to the progression of non-alcoholic steatohepatitis in humans and mice. *J Hepatol* 2012; **57**: 837-843 [PMID: 22641095 DOI: 10.1016/j.jhep.2012.05.013]

68   **Maliken BD**, Nelson JE, Klintworth HM, Beauchamp M, Yeh MM, Kowdley KV. Hepatic reticuloendothelial system cell iron deposition is associated with increased apoptosis in nonalcoholic fatty liver disease. *Hepatology* 2013; **57**: 1806-1813 [PMID: 23325576 DOI: 10.1002/hep.26238]

69   **Yang L**, Roh YS, Song J, Zhang B, Liu C, Loomba R, Seki E. Transforming growth factor beta signaling in hepatocytes participates in steatohepatitis through regulation of cell death and lipid metabolism in mice. *Hepatology* 2014; **59**: 483-495 [PMID: 23996730 DOI: 10.1002/hep.26698]

70   **Liang S**, Kisseleva T, Brenner DA. The Role of NADPH Oxidases (NOXs) in Liver Fibrosis and the Activation of Myofibroblasts. *Front Physiol* 2016; **7**: 17 [PMID: 26869935 DOI: 10.3389/fphys.2016.00017]

71   **Park S**, Kim JW, Yun H, Choi SJ, Lee SH, Choi KC, Lim CW, Lee K, Kim B. Mainstream cigarette smoke accelerates the progression of nonalcoholic steatohepatitis by modulating Kupffer cell-mediated hepatocellular apoptosis in adolescent mice. *Toxicol Lett* 2016; **256**: 53-63 [PMID: 27180087 DOI: 10.1016/j.toxlet.2016.05.012]

72   **Mann JP**, Raponi M, Nobili V. Clinical implications of

understanding the association between oxidative stress and pediatric NAFLD. *Expert Rev Gastroenterol Hepatol* 2017; **11**: 371-382 [PMID: 28162008 DOI: 10.1080/17474124.2017.1291340]

73   **Kuo TF**, Tatsukawa H, Kojima S. New insights into the functions and localization of nuclear transglutaminase 2. *FEBS J* 2011; **278**: 4756-4767 [PMID: 22051117 DOI: 10.1111/j.1742-4658.2011.08409.x]

74   **Toriguchi K**, Hatano E, Tanabe K, Takemoto K, Nakamura K, Koyama Y, Seo S, Taura K, Uemoto S. Attenuation of steatohepatitis, fibrosis, and carcinogenesis in mice fed a methionine-choline deficient diet by CCAAT/enhancer-binding protein homologous protein deficiency. *J Gastroenterol Hepatol* 2014; **29**: 1109-1118 [PMID: 24329600 DOI: 10.1111/jgh.12481]

75   **Yoo W**, Noh KH, Ahn JH, Yu JH, Seo JA, Kim SG, Choi KM, Baik SH, Choi DS, Kim TW, Kim HJ, Kim NH. HIF-1α expression as a protective strategy of HepG2 cells against fatty acid-induced toxicity. *J Cell Biochem* 2014; **115**: 1147-1158 [PMID: 24402912 DOI: 10.1002/jcb.24757]

76   **Miyamoto Y**, Mauer AS, Kumar S, Mott JL, Malhi H. Mmu-miR-615-3p regulates lipoapoptosis by inhibiting C/EBP homologous protein. *PLoS One* 2014; **9**: e109637 [PMID: 25314137 DOI: 10.1371/journal.pone.0109637]

77   **Willy JA**, Young SK, Stevens JL, Masuoka HC, Wek RC. CHOP links endoplasmic reticulum stress to NF-κB activation in the pathogenesis of nonalcoholic steatohepatitis. *Mol Biol Cell* 2015; **26**: 2190-2204 [PMID: 25904325 DOI: 10.1091/mbc.E15-01-0036]

78   **Ao N**, Yang J, Wang X, Du J. Glucagon-like peptide-1 preserves non-alcoholic fatty liver disease through inhibition of the endoplasmic reticulum stress-associated pathway. *Hepatol Res* 2016; **46**: 343-353 [PMID: 26147696 DOI: 10.1111/hepr.12551]

79   **Liu X**, Henkel AS, LeCuyer BE, Schipma MJ, Anderson KA, Green RM. Hepatocyte X-box binding protein 1 deficiency increases liver injury in mice fed a high-fat/sugar diet. *Am J Physiol Gastrointest Liver Physiol* 2015; **309**: G965-G974 [PMID: 26472223 DOI: 10.1152/ajpgi.00132.2015]

80   **Mota M**, Banini BA, Cazanave SC, Sanyal AJ. Molecular mechanisms of lipotoxicity and glucotoxicity in nonalcoholic fatty liver disease. *Metabolism* 2016; **65**: 1049-1061 [PMID: 26997538 DOI: 10.1016/j.metabol.2016.02.014]

81   **Tu QQ**, Zheng RY, Li J, Hu L, Chang YX, Li L, Li MH, Wang RY, Huang DD, Wu MC, Hu HP, Chen L, Wang HY. Palmitic acid induces autophagy in hepatocytes via JNK2 activation. *Acta Pharmacol Sin* 2014; **35**: 504-512 [PMID: 24608675 DOI: 10.1038/aps.2013.170]

82   **Cai N**, Zhao X, Jing Y, Sun K, Jiao S, Chen X, Yang H, Zhou Y, Wei L. Autophagy protects against palmitate-induced apoptosis in hepatocytes. *Cell Biosci* 2014; **4**: 28 [PMID: 24904743 DOI: 10.1186/2045-3701-4-28]

83   **Xie F**, Jia L, Lin M, Shi Y, Yin J, Liu Y, Chen D, Meng Q. ASPP2 attenuates triglycerides to protect against hepatocyte injury by reducing autophagy in a cell and mouse model of non-alcoholic fatty liver disease. *J Cell Mol Med* 2015; **19**: 155-164 [PMID: 25256142 DOI: 10.1111/jcmm.12364]

84   **Lou J**, Wang Y, Wang X, Jiang Y. Uncoupling protein 2 regulates palmitic acid-induced hepatoma cell autophagy. *Biomed Res Int* 2014; **2014**: 810401 [PMID: 25512910 DOI: 10.1155/2014/810401]

85   **Cazanave SC**, Sanyal AJ. KEAP the balance between life and death. *Mol Cell Oncol* 2014; **2**: e968065 [PMID: 27308418 DOI: 10.4161/23723548.2014.968065]

86   **Eid N**, Ito Y, Otsuki Y. Triggering of Parkin Mitochondrial Translocation in Mitophagy: Implications for Liver Diseases. *Front Pharmacol* 2016; **7**: 100 [PMID: 27199746 DOI: 10.3389/fphar.2016.00100]

87   **Tanaka S**, Hikita H, Tatsumi T, Sakamori R, Nozaki Y, Sakane S, Shiode Y, Nakabori T, Saito Y, Hiramatsu N, Tabata K, Kawabata T, Hamasaki M, Eguchi H, Nagano H, Yoshimori T, Takehara T. Rubicon inhibits autophagy and accelerates hepatocyte apoptosis

and lipid accumulation in nonalcoholic fatty liver disease in mice. *Hepatology* 2016; **64**: 1994-2014 [PMID: 27637015 DOI: 10.1002/hep.28820]

88   **Li S**, Dou X, Ning H, Song Q, Wei W, Zhang X, Shen C, Li J, Sun C, Song Z. Sirtuin 3 acts as a negative regulator of autophagy dictating hepatocyte susceptibility to lipotoxicity. *Hepatology* 2017; **66**: 936-952 [PMID: 28437863 DOI: 10.1002/hep.29229]

89   **García-Monzón C**, Lo Iacono O, Mayoral R, González-Rodríguez A, Miquilena-Colina ME, Lozano-Rodríguez T, García-Pozo L, Vargas-Castrillón J, Casado M, Boscá L, Valverde AM, Martín-Sanz P. Hepatic insulin resistance is associated with increased apoptosis and fibrogenesis in nonalcoholic steatohepatitis and chronic hepatitis C. *J Hepatol* 2011; **54**: 142-152 [PMID: 20888662 DOI: 10.1016/j.jhep.2010.06.021]

90   **Van Rooyen DM**, Larter CZ, Haigh WG, Yeh MM, Ioannou G, Kuver R, Lee SP, Teoh NC, Farrell GC. Hepatic free cholesterol accumulates in obese, diabetic mice and causes nonalcoholic steatohepatitis. *Gastroenterology* 2011; **141**: 1393-1403, 1403.e1-1403.e5 [PMID: 21703998 DOI: 10.1053/j.gastro.2011.06.040]

91   **Giannini C**, Feldstein AE, Santoro N, Kim G, Kursawe R, Pierpont B, Caprio S. Circulating levels of FGF-21 in obese youth: associations with liver fat content and markers of liver damage. *J Clin Endocrinol Metab* 2013; **98**: 2993-3000 [PMID: 23626003 DOI: 10.1210/jc.2013-1250]

92   **Li H**, Fang Q, Gao F, Fan J, Zhou J, Wang X, Zhang H, Pan X, Bao Y, Xiang K, Xu A, Jia W. Fibroblast growth factor 21 levels are increased in nonalcoholic fatty liver disease patients and are correlated with hepatic triglyceride. *J Hepatol* 2010; **53**: 934-940 [PMID: 20675007 DOI: 10.1016/j.jhep.2010.05.018]

93   **Williams KH**, Vieira De Ribeiro AJ, Prakoso E, Veillard AS, Shackel NA, Brooks B, Bu Y, Cavanagh E, Raleigh J, McLennan SV, McCaughan GW, Keane FM, Zekry A, Gorrell MD, Twigg SM. Circulating dipeptidyl peptidase-4 activity correlates with measures of hepatocyte apoptosis and fibrosis in non-alcoholic fatty liver disease in type 2 diabetes mellitus and obesity: A dual cohort cross-sectional study. *J Diabetes* 2015; **7**: 809-819 [PMID: 25350950 DOI: 10.1111/1753-0407.12237]

94   **Kondo Y**, Ishigami A. Involvement of senescence marker protein-30 in glucose metabolism disorder and non-alcoholic fatty liver disease. *Geriatr Gerontol Int* 2016; **16** Suppl 1: 4-16 [PMID: 27018279 DOI: 10.1111/ggi.12722]

95   **Idrissova L**, Malhi H, Werneburg NW, LeBrasseur NK, Bronk SF, Fingas C, Tchkonia T, Pirtskhalava T, White TA, Stout MB, Hirsova P, Krishnan A, Liedtke C, Trautwein C, Finnberg N, El-Deiry WS, Kirkland JL, Gores GJ. TRAIL receptor deletion in mice suppresses the inflammation of nutrient excess. *J Hepatol* 2015; **62**: 1156-1163 [PMID: 25445398 DOI: 10.1016/j.jhep.2014.11.033]

96   **Cartland SP**, Harith HH, Genner SW, Dang L, Cogger VC, Vellozzi M, Di Bartolo BA, Thomas SR, Adams LA, Kavurma MM. Non-alcoholic fatty liver disease, vascular inflammation and insulin resistance are exacerbated by TRAIL deletion in mice. *Sci Rep* 2017; **7**: 1898 [PMID: 28507343 DOI: 10.1038/s41598-017-01721-4]

97   **Machado MV**, Cortez-Pinto H. Cell death and nonalcoholic steatohepatitis: where is ballooning relevant? *Expert Rev Gastroenterol Hepatol* 2011; **5**: 213-222 [PMID: 21476916 DOI: 10.1586/egh.11.6]

98   **Csak T**, Ganz M, Pespisa J, Kodys K, Dolganiuc A, Szabo G. Fatty acid and endotoxin activate inflammasomes in mouse hepatocytes that release danger signals to stimulate immune cells. *Hepatology* 2011; **54**: 133-144 [PMID: 21488066 DOI: 10.1002/hep.24341]

99   **Henao-Mejia J**, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, Thaiss CA, Kau AL, Eisenbarth SC, Jurczak MJ, Camporez JP, Shulman GI, Gordon JI, Hoffman HM, Flavell RA. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. *Nature* 2012; **482**: 179-185 [PMID: 22297845 DOI: 10.1038/nature10809]

100   **Koutsounas I**, Giaginis C, Theocharis S. Farnesoid X Receptor (FXR) from normal to malignant state. *Histol Histopathol* 2012;

27: 835-853 [PMID: 22648540 DOI: 10.14670/HH-27.835]

101   **Akazawa Y**, Guicciardi ME, Cazanave SC, Bronk SF, Werneburg NW, Kakisaka K, Nakao K, Gores GJ. Degradation of cIAPs contributes to hepatocyte lipoapoptosis. *Am J Physiol Gastrointest Liver Physiol* 2013; **305**: G611-G619 [PMID: 24008361 DOI: 10.1152/ajpgi.00111.2013]

102   **Egnatchik RA**, Leamy AK, Jacobson DA, Shiota M, Young JD. ER calcium release promotes mitochondrial dysfunction and hepatic cell lipotoxicity in response to palmitate overload. *Mol Metab* 2014; **3**: 544-553 [PMID: 25061559 DOI: 10.1016/j.molmet.2014.05.004]

103   **Gan LT**, Van Rooyen DM, Koina ME, McCuskey RS, Teoh NC, Farrell GC. Hepatocyte free cholesterol lipotoxicity results from JNK1-mediated mitochondrial injury and is HMGB1 and TLR4-dependent. *J Hepatol* 2014; **61**: 1376-1384 [PMID: 25064435 DOI: 10.1016/j.jhep.2014.07.024]

104   **Zhu C**, Xie P, Zhao F, Zhang L, An W, Zhan Y. Mechanism of the promotion of steatotic HepG2 cell apoptosis by cholesterol. *Int J Clin Exp Pathol* 2014; **7**: 6807-6813 [PMID: 25400762]

105   **Seeße J**, Liebisch G, Schmitz G, Stremmel W, Chamulitrat W. Palmitate activation by fatty acid transport protein 4 as a model system for hepatocellular apoptosis and steatosis. *Biochim Biophys Acta* 2015; **1851**: 549-565 [PMID: 25603556 DOI: 10.1016/j.bbalip.2015.01.004]

106   **Denechaud PD**, Lopez-Mejia IC, Giralt A, Lai Q, Blanchet E, Delacuisine B, Nicolay BN, Dyson NJ, Bonner C, Pattou F, Annicotte JS, Fajas L. E2F1 mediates sustained lipogenesis and contributes to hepatic steatosis. *J Clin Invest* 2016; **126**: 137-150 [PMID: 26619117 DOI: 10.1172/JCI81542]

107   **Baranova A**, Tran TP, Afendy A, Wang L, Shamsaddini A, Mehta R, Chandhoke V, Birerdinc A, Younossi ZM. Molecular signature of adipose tissue in patients with both non-alcoholic fatty liver disease (NAFLD) and polycystic ovarian syndrome (PCOS). *J Transl Med* 2013; **11**: 133 [PMID: 23721173 DOI: 10.1186/1479-5876-11-133]

108   **Rodrigues PM**, Afonso MB, Simão AL, Carvalho CC, Trindade A, Duarte A, Borralho PM, Machado MV, Cortez-Pinto H, Rodrigues CM, Castro RE. miR-21 ablation and obeticholic acid ameliorate nonalcoholic steatohepatitis in mice. Cell Death Dis 2017; 8: e2748 [PMID: 28406477 DOI: 10.1038/cddis.2017.172]

109   **Kirsch R**, Clarkson V, Verdonk RC, Marais AD, Shephard EG, Ryffel B, de la M Hall P. Rodent nutritional model of steatohepatitis: effects of endotoxin (lipopolysaccharide) and tumor necrosis factor alpha deficiency. *J Gastroenterol Hepatol* 2006; **21**: 174-182 [PMID: 16706830]

110   **Kudo H**, Takahara T, Yata Y, Kawai K, Zhang W, Sugiyama T. Lipopolysaccharide triggered TNF-alpha-induced hepatocyte apoptosis in a murine non-alcoholic steatohepatitis model. *J Hepatol* 2009; **51**: 168-175 [PMID: 19446916 DOI: 10.1016/j.jhep.2009.02.032]

111   **Hirsova P**, Ibrahim SH, Gores GJ, Malhi H. Lipotoxic lethal and sublethal stress signaling in hepatocytes: relevance to NASH pathogenesis. *J Lipid Res* 2016; **57**: 1758-1770 [PMID: 27049024]

112   **Dechandt CRP**, Zuccolotto-Dos-Reis FH, Teodoro BG, Fernandes AMAP, Eberlin MN, Kettelhut IC, Curti C, Alberici LC. Triacsin C reduces lipid droplet formation and induces mitochondrial biogenesis in primary rat hepatocytes. *J Bioenerg Biomembr* 2017; **49**: 399-411 [PMID: 28918598 DOI: 10.1007/s10863-017-9725-9]

113   **Kim SH**, Kim G, Han DH, Lee M, Kim I, Kim B, Kim KH, Song YM, Yoo JE, Wang HJ, Bae SH, Lee YH, Lee BW, Kang ES, Cha BS, Lee MS. Ezetimibe ameliorates steatohepatitis via AMP activated protein kinase-TFEB-mediated activation of autophagy and NLRP3 inflammasome inhibition. *Autophagy* 2017; **13**: 1767-1781 [PMID: 28933629 DOI: 10.1080/15548627.2017.1356977]

114   **Zhang J**, Zhang H, Deng X, Zhang N, Liu B, Xin S, Li G, Xu K. Baicalin attenuates non-alcoholic steatohepatitis by suppressing key regulators of lipid metabolism, inflammation and fibrosis in mice. *Life Sci* 2018; **192**: 46-54 [PMID: 29158052 DOI: 10.1016/j.lfs.2017.11.027]

115   **Nam HH**, Jun DW, Jang K, Saeed WK, Lee JS, Kang HT, Chae

YJ. Granulocyte colony stimulating factor treatment in non-alcoholic fatty liver disease: beyond marrow cell mobilization. *Oncotarget* 2017; **8**: 97965-97976 [PMID: 29228666 DOI: 10.18632/oncotarget.18967]

116   **Issa D**, Wattacheril J, Sanyal AJ. Treatment options for nonalcoholic steatohepatitis - a safety evaluation. *Expert Opin Drug Saf* 2017; **16**: 903-913 [PMID: 28641031 DOI: 10.1080/147 40338.2017.1343299]

117   **Huang XL**, He Y, Ji LL, Wang KY, Wang YL, Chen DF, Geng Y, OuYang P, Lai WM. Hepatoprotective potential of isoquercitrin against type 2 diabetes-induced hepatic injury in rats. *Oncotarget* 2017; **8**: 101545-101559 [PMID: 29254185 DOI: 10.18632/oncotarget.21074]

118   **Shi T**, Yang X, Zhou H, Xi J, Sun J, Ke Y, Zhang J, Shao Y, Jiang X, Pan X, Liu S, Zhuang R. Activated carbon N-acetylcysteine microcapsule protects against nonalcoholic fatty liver disease in young rats via activating telomerase and inhibiting apoptosis. *PLoS One* 2018; **13**: e0189856 [PMID: 29324774 DOI: 10.1371/journal.pone.0189856]

119   **Ou-Yang Q**, Xuan CX, Wang X, Luo HQ, Liu JE, Wang LL, Li TT, Chen YP, Liu J. 3-Acetyl-oleanolic acid ameliorates non-alcoholic fatty liver disease in high fat diet-treated rats by activating AMPK-related pathways. *Acta Pharmacol Sin* 2018; Epub ahead of print [PMID: 29345253 DOI: 10.1038/aps.2017.142]

120   **Huang F**, Wang J, Yu F, Tang Y, Ding G, Yang Z, Sun Y. Protective Effect of Meretrix meretrix Oligopeptides on High-Fat-Diet-Induced Non-Alcoholic Fatty Liver Disease in Mice. *Mar Drugs* 2018; **16**: pii: E39 [PMID: 29360762 DOI: 10.3390/md16020039]

121   **Haczeyni F**, Wang H, Barn V, Mridha AR, Yeh MM, Haigh WG, Ioannou GN, Choi YJ, McWherter CA, Teoh NC, Farrell GC. The selective peroxisome proliferator-activated receptor-delta agonist seladelpar reverses nonalcoholic steatohepatitis pathology by abrogating lipotoxicity in diabetic obese mice. *Hepatol Commun* 2017; **1**: 663-674 [PMID: 29404484 DOI: 10.1002/hep4.1072]

122   **Hajighasem A**, Farzanegi P, Mazaheri Z. Effects of combined therapy with resveratrol, continuous and interval exercises on apoptosis, oxidative stress, and inflammatory biomarkers in the liver of old rats with non-alcoholic fatty liver disease. *Arch Physiol Biochem* 2018; 1-8 [PMID: 29463133 DOI: 10.1080/13813455.20 18.1441872]

123   **Sharma RS**, Harrison DJ, Kisielewski D, Cassidy DM, McNeilly AD, Gallagher JR, Walsh SV, Honda T, McCrimmon RJ, Dinkova-Kostova AT, Ashford MLJ, Dillon JF, Hayes JD. Experimental Nonalcoholic Steatohepatitis and Liver Fibrosis Are Ameliorated by Pharmacologic Activation of Nrf2 (NF-E2 p45-Related Factor 2). *Cell Mol Gastroenterol Hepatol* 2017; **5**: 367-398 [PMID: 29552625 DOI: 10.1016/j.jcmgh.2017.11.016]

124   **Nakamura M**, Kanda T, Sasaki R, Haga Y, Jiang X, Wu S, Nakamoto S, Yokosuka O. MicroRNA-122 Inhibits the Production of Inflammatory Cytokines by Targeting the PKR Activator PACT in Human Hepatic Stellate Cells. *PLoS One* 2015; **10**: e0144295 [PMID: 26636761 DOI: 10.1371/journal.pone.0144295]

125   **Sumida Y**, Yoneda M. Current and future pharmacological therapies for NAFLD/NASH. *J Gastroenterol* 2018; **53**: 362-376 [PMID: 29247356 DOI: 10.1007/s00535-017-1415-1]

126   **Jung Y**, Diehl AM. Non-alcoholic steatohepatitis pathogenesis: role of repair in regulating the disease progression. *Dig Dis* 2010; **28**: 225-228 [PMID: 20460916 DOI: 10.1159/000282092]

REVIEW

Diets, functional foods, and nutraceuticals as alternative therapies for inflammatory bowel disease: Present status and future trends

Mohammad Al Mijan, Beong Ou Lim

Mohammad Al Mijan, Beong Ou Lim, Department of Integrated Biosciences, College of Biomedical & Health Science, Konkuk University, Chungju 380-701, South Korea

ORCID number: Mohammad Al Mijan (0000-0003-3530-4699); Beong Ou Lim (0000-0002-9618-5956).

Author contributions: Mijan MA prepared the manuscript; Lim BO provided overall guidance and supervision in writing the article.

Conflict-of-interest statement: The authors declare no conflict-of-interests.

Manuscript source: Invited manuscript

Correspondence to: Beong Ou Lim, PhD, Professor, Department of Integrated Biosciences, College of Biomedical & Health Science, Konkuk University, 322 Danwol-dong, Chungju-shi, Chungbuk-do 380-701, South Korea. beongou@kku.ac.kr
Telephone: +82-43- 8403570
Fax: +82-43- 8563572

Abstract

Inflammatory bowel disease (IBD) is a serious health concern among western societies. The disease is also on the rise in some East Asian countries and in Australia. Health professionals and dietitians around the world are facing an unprecedented challenge to prevent and control the increasing prevalence of IBD. The current therapeutic strategy that includes drugs and biological treatments is inefficient and are associated with adverse health consequences. In this context, the use of natural products is gaining worldwide attention. *In vivo* studies and clinical evidence suggest that well-planned dietary regimens with specific nutrients can alleviate gastrointestinal inflammation by modulating inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin 1 (IL-1), IL-6, IL-1β, and IL-10. Alternatively, the avoidance of high-fat and high-carbohydrate diets is regarded as an effective tool to eliminate the causes of IBD. Many functional foods and bioactive components have received attention for showing strong therapeutic effects against IBD. Both animal and human studies suggest that bioactive functional foods can ameliorate IBD by downregulating the pro-inflammatory signaling pathways, such as nuclear factor κB, STAT1, STAT6, and pro-inflammatory cytokines, including IL-1β, IL-4, IL-6, COX-2, TNF-α, and interferon γ. Therefore, functional foods and diets have the potential to alleviate IBD by modulating the underlying pathogenic mechanisms. Future comprehensive studies are needed to corroborate the potential roles of functional foods and diets in the prevention and control of IBD.

Key words: Inflammatory bowel disease; Colitis; Diets; Functional foods; Bioactive compounds; Inflammatory cytokines; Alternative therapy

Core tip: Diets and functional foods are two of the most potential alternative therapies for inflammatory bowel disease (IBD). Dietary supplementation of probiotics and non-starch polysaccharides demonstrated strong therapeutic actions on IBD. Likewise, functional foods have received more attention than ever as alternative therapies for IBD. Plant-derived extracts and bioactive compounds exhibited anti-inflammatory actions against IBD. Both diets and functional foods have a very important role to play in the near future. We have discussed the roles of both diets and functional foods in IBD management.

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic gastrointestinal disorder characterized by relapsing inflammation and severe mucosal damage in the intestine. There are two common forms of IBD, namely, ulcerative colitis (UC) and Crohn's disease (CD), which are generally associated with diarrhea, nausea, abdominal pain, fatigue, rectal bleeding, weight loss, anxiety, *etc*[1]. Currently, IBD is one of the most prevalent gastrointestinal diseases among the developed nations in the West, affecting nearly 1.6 million people in the United States and 2.5-3.0 million people in northern Europe[2,3]. Although IBD is mostly prevalent in North America and Europe, the adoption of western dietary habits and lifestyle has led to, countries like- China, South Korea, and Australia witnessing a significant rise in the incidence of IBD[4].

The exact etiology of IBD has yet to be defined, but it is believed that genetic susceptibility, environment, immunoregulatory dysfunction, intestinal microbiota, nutrition, and lifestyle are the key players in the pathogenesis of IBD[5]. Activation of macrophages and an uncontrolled production of pro- and anti-inflammatory cytokines, including tumor necrosis factor α (TNF-α), interleukins, and interferon γ (IFN-γ) in the intestinal mucosa, mediate the inflammation by inducing inflammatory pathways[6]. Conventional therapies based on steroidal and non-steroidal drugs and biological agents are inefficient in treating IBD and are often associated with adverse side effects. As a consequence, tremendous research attention is now being focused on finding alternative therapies based on plants and other natural products.

There is a growing consensus that diet and nutrition play a critical role in the etiopathogenesis of IBD, and hence dietary therapy has a great implication on the treatment of IBD[7]. Recent research evidence suggests that the supplementation of fruits and vegetables, probiotic bacteria, dietary fibers, and fat-soluble vitamins can substantially reduce the symptoms of IBD through their anti-inflammatory functions[7-11]. In contrast, as the high-fat and high-carbohydrate foods are supposedly involved in the etiology of IBD, eliminating these foods from the diet could be an essential tool in the management of IBD[12]. Bioactive natural compounds and functional foods have been a major focus of research throughout the last decade as potential therapies for IBD, and many research groups have demonstrated positive and outstanding results. Plant-derived extracts, antioxidants, phytochemicals, polyunsaturated fatty acids, and dietary peptides have demonstrated strong anti-inflammatory effects against IBD due to their modulatory actions on pro- and anti-inflammatory cytokines and signaling pathways[13-18]. Ongoing and future research is expected to provide more evidence and explanations regarding the use of diets and functional foods to control IBD. It appears that the alternative therapies based on diets and functional foods will be the future of IBD management. The present study therefore provides an overview on the current status and the future direction of the use of diets, functional foods, and bioactive compounds against IBD.

CURRENT STATUS AND TREATMENTS OF IBD

The chronic and recurrent inflammation of the gastrointestinal tract associated with IBD, represented by UC and CD accompanies several gastrointestinal and systemic disorders and mental illnesses[19]. Although the two forms of IBD share some common features, they are regarded as separate entities, as they possess distinct histopathological and symptomatic characteristics. UC is generally defined as a mucosal or submucosal inflammation of mainly the rectum and occasionally of the colonic area. The common symptoms of UC include abdominal pain, diarrhea, malnutrition, rectal pain and bleeding. CD is regarded as a transmural inflammation of the ileum and colon, though it can affect any part of the gastrointestinal tract and form granulomas, fistulas, and strictures in the intestine. Patients diagnosed with CD often have abdominal pain, diarrhea, fever, loss of appetite and weight, anemia, and intermittent anal fissures.

Although the exact etiology of IBD has yet to be specified, a number of factors including- diet, immunity, environment, heredity, and microbiota, contribute to the development of IBD[20]. A complex interaction of environmental, genetic, microbial, and immunological factors might cause the activation of the mucosal immune response and the release of numerous cytokines[21]. Cytokines are cell signaling molecules generated predominantly by immune cells that have specific roles in the communication and interaction between cells and

Table 1 Overview of the conventional therapies for inflammatory bowel disease

Therapeutic agent	Active compound	Mode of action	Ref.
Aminosalicylates (ASA)	5-ASA	Decreases MPO activity, inhibits β-catenin activation	[23]
		Inhibits the generation and activity of IL-1β, IL-4, IL-5, IL-8, granulocyte-macrophage colony stimulating factor, and TNF-α	
Corticosteroids	Corticosteroids		[24]
Immunosuppressants	Azathioprine	Clinical remission	[25]
	6-mercaptopurine	Mucosal healing	
	Cyclosporine A		
	Tacrolimus		
	Methotrexaten		
Antibiotics	Metronidazole	Decrease disease activity index	[26]
	Ciprofloxacin	Maintain remission	
Biological therapy	Infliximab	Neutralizes TNF-α	[27]
	Adalimumab	Reduces inflammation	
	Certolizumab		

TNF-α: Tumor necrosis factor α; IL: Interleukin.

the onset of local and systemic inflammation. Under normal conditions, the intestinal mucosa can maintain the balance between pro-inflammatory cytokines, such as TNF-α, IFN-γ, interleukin (IL)-1, IL-6, and IL-12 and anti-inflammatory cytokines, which includes IL-4, IL-10, and IL-11. In IBD patients, the intestinal homeostasis and the fine balance between pro- and anti-inflammatory cytokines is disrupted, causing an increased number and activities of pro-inflammatory cytokines in the mucosa, leading to tissue damage and inflammation. Furthermore, the weakened epithelial barrier function and the increased intestinal permeability in IBD subjects facilitate mucosal inflammation[22].

Currently, there is no effective therapy available that can completely cure IBD. Current therapeutic options are incapable of targeting the underlying pathogenic mechanisms of IBD; instead, they are specifically designed to instigate and maintain the remission of the disease and help mitigate complications in patients[1]. Aminosalicylates and corticosteroids are considered first-line therapy for IBD (Table 1). Both of these drugs have shown efficacies in ameliorating the severity and the symptoms of IBD through their abilities to down-regulate the pro-inflammatory cytokines and signaling pathways[23,24]. Immunosuppressive agents, including azathioprine, 6-mercaptopurine, cyclosporine A, and antibiotics, which are mostly used as adjunct therapies, can decrease intestinal inflammation by suppressing the mucosal immune response[25,26] (Table 1). A more recent and innovative approach is called "biological therapy," where monoclonal antibodies, such as infliximab and adalimumap, are applied to downregulate the immune response pathways[27].

Despite providing some symptomatic and temporary relief, current drug therapies are described as inadequate with serious side effects[28]. Biological therapies, which are currently a mainstay for of IBD treatment, are expensive and associated with adverse health effects.

Therefore, the development of alternative IBD therapies using natural products that are highly effective, safe, and inexpensive is in great demand.

DIETS AND DIETARY INTERVENTIONS FOR IBD

Diets comprise the usual food and drink that a person regularly consume. Diets, among other factors, play a crucial role in the etiology of IBD. Dietary interventions in the form of either providing specific nutrients or dietary restrictions are regarded as effective tools in treating IBD. Currently, due to the lack of adequate data and research evidence, health professionals and dieticians often find it difficult to recommend dietary strategies for IBD patients. However, recent research outcomes are providing evidence that many nutrients and food elements can cure IBD symptoms; hence a dietary plan based on proper nutrients could be an effective therapeutic strategy against IBD.

Probiotics

Probiotics are described as live microorganisms that benefit humans by promoting gut health and the immune system upon ingestion in an acceptable amount. Numerous possible mechanisms through which probiotic bacteria exert their beneficial effects have been proposed. Probiotics can reduce harmful microorganisms and maintain the microbial balance inside the gut by blocking the site of adhesion, competing for nutrients, and killing pathogenic microorganisms[29]. Production of short-chain fatty acids (SCFA) and butyrate by probiotic bacteria lowers the pH level in the colon and limits the growth of pathogens[30]. In addition, probiotic bacteria can function as anti-inflammatory agents by modulating the NF-κB signaling pathway, inflammatory cytokines, and the regulatory T cell response[31]. Two of

the most widely studied genera that have been proven effective in alleviating gastrointestinal inflammation are *Lactobacillus* and *Bifidobacteria*. Lee *et al*[32] reported that *Lactobacillus suntoryeus* suppressed toll-like receptor (TLR)-4 linked NF-κB and IL-6 expression in TNBS-induced colitis (Table 2). In a mouse model of IBD induced by *E. coli* 0111 LPS, soy milk fermented with *Lactococcus lactis* subsp. *lactis* S-SU2 prevented colonic shortening and spleen enlargement, and repaired epithelial damage[33]. *Lactobacillus paracasei* LS2 isolated from kimchi decreased the number of neutrophils (CD11b$^+$Gr-1$^+$) and, macrophages (CD11b$^+$ F4/80$^+$), and decreased TNF-α and IFN-γ expression in DSS-induced UC[34]. An oral administration of *Lactococcus lactis* NZ9000 (NZ-HO) secreting an anti-inflammatory substance called recombinant mouse heme oxygenase (mHO-1) to mice decreased the disease activity index (DAI), increased the production of IL-10, and suppressed IL-1α and IL-6 expression[35]. A study by Yokota *et al*[36] revealed that supplying drinking water containing *Lactobacillus plantarum* AN1 isolated from fermented fish to an IBD mouse model increased the indigenous population of lactic acid bacteria in the colon, and their synergistic effects reversed colonic shortening, spleen enlargement, and colonic tissue damage significantly. Several other strains of *Lactobacillus plantarum* exhibited therapeutic effects on gastrointestinal inflammation through their modulatory functions against inflammatory cytokines[37]. A recent study indicated that *Lactobacillus sakei* attenuated the clinical symptoms and histological damage by suppressing inflammatory mediators, such as NF-κB, STAT1, and TL4[38]. A combined therapy consisting of *Lactobacillus casei,* butyrate, and *Pistacia atlantica* significantly improved histological scores and reduced MPO activity in a rat model of IBD[39].

The antimicrobial and anti-inflammatory effects of *Bifidobacteria* are also well-known, and this probiotic genus has a wide application against gastrointestinal inflammation. Reportedly, *Bifidobacterium adolescentis* IM38 alleviated inflammation by downregulating NF-κB expression and lipopolysaccharide production in high-fat diet-induced ulcerative colitis in mice[40]. An *in vitro* and *in vivo* study suggested that *Bifidobacteria bifidum* 231 enhanced the IL-10 production in IEC-6 cell lines and improved the macroscopic and histological conditions in TNBS-induced colitis[41]. *Bifidobacterium longum* CCM7952 strengthened the epithelial barrier function and reduced clinical symptoms in experimental colitis[31].

Non-starch polysaccharides
Non-starch polysaccharides (NPS), classified as dietary fiber and prebiotics, are obtained from various natural sources that have been studied extensively as therapeutics against inflammation and other immune-related problems. All of the major components of NPS, including cellulose, glucomannan, glucan, pectin, inulin, and oligosaccharides have exhibited anti-inflammatory and immunomodulatory functions[42]. It has been suggested that most of the NPS components reach the large intestine intact, where they are fermented by probiotic and useful bacteria to exert their anti-inflammatory functions[43].

Konjac glucomannan is a plant-derived polysaccharide that has been used to treat gastrointestinal inflammatory disorders. For example, supplementation with konjac glucomannan hydrolysate for fourteen days to IBD patients resulted in improved bowel movement, fecal consistence, reduced abdominal pain, and a better lifestyle[44] (Table 2). β-Glucan was orally administered to an animal model of IBD, which resulted in improved fecal output and reduced colorectal distension[45]. In another study, oat β-glucan reduced the levels of MPO, NO, and MDA, and suppressed the expression of IL-1β, IL-6, and iNOS in a DSS-induced colitis model in mice[46]. Bacterial β-(1,3)-glucan prevented IBD in mice by recovering regulatory T cells (Tregs) and the defects of natural killer (NK) cells and by suppressing the excessive production of IgA[47,48]. Azuma *et al*[49,50] reported that cellulose nanofibers obtained from seaweed and pear reversed colonic shortening, reduced colonic damage and, suppressed NF-κB expression and MPO activity in colitic mice.

Prebiotics are non-digestible polysaccharides, which generally includes oligosaccharides and inulin that act as nutrients for the native gut microbiota that offer health benefits to the host[30]. Providing fructooligosaccharides to colitic mice resulted in increased lactic acid bacteria in the gut and decreased pro-inflammatory cytokines such as, IFN-γ, IL-17, and TNF-α[51]. In a DSS-induced colitis mouse model, goat milk oligosaccharides reduced the colonic tissue damage and increased the favo-rable microbial population in the intestine[52]. Štofilová *et al*[53] demonstrated that prebiotic inulin together with *Lactobacillus plantarum* LS/07 CCM7766 symbiotically improved colonic and jejunal tissue damage by down-regulating IL-2, IL-6, IL-17, TNF-α, COX-2, and NF-κB expression in an N, N-dimethylhydrazin-induced colitis model in rats.

Vitamins
Fat soluble vitamins such as Vitamin A and D have protec-tive roles against the pathogenesis of IBD. Accumulating evidence suggests that patients with IBD are frequently diagnosed with low levels of fat soluble vitamins, and therefore specific supplementations of those vitamins are often recommended[5]. Gubatan *et al*[54] recently reported that a low level of vitamin D in the patients with UC at the time of remission increased the risk of clinical relapse of IBD. Vitamin D deficiency in UC patients has been found to be associated with mucosal inflammation and disease activity[55]. Therefore, vitamin D supplementation can result in positive outcomes in IBD patients. It has been demonstrated that vitamin D, by downregulating pro-inflammatory cytokines, IL-6, IL-21, TNF-α, and IFN-γ and by stabilizing the intestinal barrier, can contribute to the amelioration of IBD symptoms[56]. According to Zhu

Table 2 Role of nutrients and diets against inflammatory bowel disease

Base material	Main compounds/agents	Mode of action	Ref.
Probiotics			
Lactic acid bacteria	*Lactobacillus suntoryeus*	Inhibited the activation of TLR-4-linked NF-κB activation	[32]
	Lactococcus lactis subsp. lactis S-SU2	Prevented the colonic shortening, lowering of liver and thymus weights, and spleen enlargement	[33]
	Lactobacillus paracasei LS2 (from kimchi)	Increased IL-10	[34]
		Reduced TNF-α, IFN-γ, IL-1β and MPO activity	
	Lactococcus lactis NZ9000 (NZ-HO)	Reduced CD11b+ F4/80+ and CD11b+ Gr-1+	[35]
	Lactobacillus plantarum AN1	Increased IL-10; reduced IL-1α and IL-6	[36]
	Lactobacillus sakei K040706	Ameliorated the atrophy of colon length, mucosal damage, and spleen enlargement	[38]
Bifidobacteria	*Bifidobacterium bifidum* 231	Reduced the expression of iNOS, TNF-α, IL-1β, and IL-6	[41]
	Bifidobacterium longum CCM7952	Suppressed NF-κB, STAT3, and TLR4 expression	[31]
		Increased IL-10; Decreased IL-1β	
		Engaged TLR2; Contained NOD2	
		Improved epithelial barrier	
Dietary fibers and prebiotics			
Konjac glucomannan	Konjac glucomannan hydrolysate	Reduced bowel movement, diarrhea, blood in feces, abdominal pain, and flatulence	[44]
Glucan	β-(1,3–1,6)-d-glucan	Improved fecal output	[45]
	Oat β-glucan	Reduced visceral pain	[46]
		Lowered MPO, NO, and MDA	
		Inhibited the expressions of TNF-α, IL-1β, IL-6 and iNOS	
	Glucan from mushroom (*Pleurotus pulmonarius*)	Reduced histological damage	[47]
	Bacterial β-(1,3)-glucan	Reduced the expression of IL-1β	[48]
		Reversed Treg reduction	
Nanofiber	Cellulose nanofiber from seaweed	Decreased NK cell defects and IgA production	[49]
Prebiotics	Cellulose nanofiber from pear	Improved intestinal tissue injury	[50]
		Suppressed the activation of NF-κB	
	Fructooligosaccharides	Suppressed colon atrophy	[51]
		Suppressed the activation of NF-κB	
	Goat milk oligosaccharide	Decreased IFN-γ, IL-17, and TNF-α levels	[52]
	Inulin	Increased LAB population	[53]
		Decreased inflammation	
		Improved mucosal damage	
		Decreased TNFα, COX-2, IL-2, and IL-6	
Vitamins	1alpha,25-dihydroxyvitamin D3	Suppressed TNF-α	[57]
		Enhanced IL-10 production	
	1,25-dihydroxyvitamin D3	Reduced IFN-γ	[58]
	Vitamin D3	Increased CD+ T cells and IL-6	[59]
		Protected mitochondria	
	Vitamin A	Inhibited nuclear respiratory factor (NFR)-1 and mitochondrial transcription factor A (TFAM)	[60]

TLR: Toll-like receptor; TNF-α: Tumor necrosis factor α; IFN-γ: Interferon γ; IL: Interleukin; NF-κB: Nuclear factor κB.

et al[57] (2005), 1α,25-dihydroxyvitamin D3 together with calcium substantially reduced TNF-α expression and relieved the symptoms of IBD in IL-10 knockout mice. In human CD4+ cells, 1,25-dihydroxyvitamin D3 increased the level of IL-10 and inhibited the proliferation of T cells[58]. Patients suffering from Crohn's disease have shown an increased level of IL-6 after vitamin D3 treatment[59]. The protective effects of vitamin A have been investigated in a TNBS-induced colitis mouse

model, and after 21 days of treatment, vitamin A substantially increased the proliferation of mitochondrial transcription factors NFR-1 and TFAM and prevented intestinal tissue damage[60].

Specific carbohydrate diets and FODMAP
The specific carbohydrate diet (SCD) was first designed and mentioned by Sydney Haas in 1914 to cure celiac disease[20]. The term became popularized when Elaine

Gottschall published his book entitled "Breaking the Vicious Cycle: intestinal health through diet" in 2012 about how he and his daughter were cured from IBD by strictly following this diet plan[61]. The SCD plan allows the intake of only monosaccharides and the complete avoidance of disaccharides and polysaccharides, because they remain undigested and unabsorbed in the digestive tract and lead to the overproduction of yeast and bacteria, which eventually causes intestinal injury. In a clinical study, children with Crohn's disease under this dietary plan for 12 and 52 wk had remarkably reduced mucosal damage and improved clinical symptoms[62]. FODMAPs are specific carbohydrate foods that contain mono-, di-, and oligosaccharides and polyols[63]. These carbohydrates are poorly digested, but easily fermented by the colonic bacteria, leading to bloating, abdominal cramping and discomfort, and diarrhea, which are also associated with IBD[64]. As a consequence, scientists developed the idea of eliminating FODMAPs from the diet for IBD patients as a cure. The efficacies of FODMAP diets have been demonstrated through human studies, and patients who adhered to FODMAP elimination have experienced fewer abdominal disorders and better quality of life[65,66].

FUNCTIONAL FOODS AND NUTRACEUTICALS FOR IBD

Functional foods are any fresh or processed foods that provide health benefits and have disease prevention activities beyond their basic nutritional value. Nutraceuticals are foods or food supplements that deliver concentrated form of bioactive substances with medicinal properties. Although functional foods have been used as traditional medicines to treat chronic diseases for several centuries, it is modern scientific discoveries that are establishing the health benefits of functional foods and natural bioactive compounds providing the underlying mechanisms of their actions. Potential roles of functional foods against IBD have been broadly studied over the last decade, and overwhelming research evidence suggests that plant extracts, polyphenols, fatty acids, and amino acids can attenuate IBD symptoms by interfering with inflammatory pathways[6,67] (Table 3).

Plant and fruit extracts

The history of using plant extracts as alternative therapies for boosting the immune system and treating chronic inflammatory disorders dates back to ancient times. The anti-inflammatory activities of the plant extracts derive mainly from their abilities to modulate inflammatory cytokines. Several plant-derived extracts have strong therapeutic effects against IBD, and there is a growing interest in developing an effective IBD therapy based on plant extracts. *Coriolus versicolor,* mostly grown in China is a medicinal mushroom that has well-known health benefits. This mushroom contains polysaccharides,

including krestin, lignin, and glucan. Our investigation on the effect of *Coriolus versicolor* extract (CVE) on UC in mice demonstrated that CVE could relieve the symptoms of colitis by decreasing the level of IgE in the serum and lymph nodes and, by suppressing the expression of TNF-α, IFN-γ, IL-4, IL-1β and IL-6[68]. *Cordyceps militaris,* a folk medicinal mushroom found in East Asia, also has proven health benefits against inflammation, most likely due to its polysaccharide contents[69]. A study by Han *et al*[70] reported that *Cordyceps militaris* prevented epithelial damage, inflammatory cell migration, and colonic shortening by decreasing TNF-α and iNOS levels. Interestingly, recent studies claim that the mushrooms grown on germinated cereal grains are rich in antioxidants and other bioactives and possess potent antioxidative and anti-inflammatory functions[71,72]. The chaga mushroom grown on germinated brown rice suppressed the expression of COX-2, TNF-α, IL-4, STAT1 and STAT6, and reduced the levels of IgE and IgA[73]. Additionally, *Ganoderma lucidum* grown on germinated rice reduced the inflammation in colitic mice by downregulating NF-κB and MAPK pathways[74].

Fruit extracts, due to their potential nutraceutical properties, have been investigated as therapeutic agents to treat IBD. A *Prunus mume* mixture in a DSS-induced colitis mouse model ameliorated inflammation by decreasing inflammatory cytokines and the immune response[75]. Pomegranate (*Punica granatum*), a tropical fruit rich in polyphenols especially ellagitannins and ellagic acid, has antioxidant and anti-inflammatory properties[76]. Pomegranate extracts, in TNBS-induced colitis rats, decreased TNF-α, MAPK phosphorylation, and NF-κB translocation[77]. Berry fruits are known for being rich in bioactive compounds and their therapeutic actions against numerous health problems[78]. The oral administration of blueberry extracts to colitic mice alleviated inflammation by a three-fold mechanism: antioxidation, inhibition of NF-κB translocation, and suppression of inflammatory cytokines[79]. The application of *Aronia melanocarpa* Elliot, also known as black chokeberry, relieved colitis symptoms in mice through its antioxidative and anti-inflammatory activities[80,81]. Ginger, which has been traditionally used as a spice and a natural remedy, has shown anti-inflammatory properties. Ginger extract, when administered to colitic mice, ameliorated colonic inflammation by downregulating NF-κB and IL-1β expression[82].

Marine foods and extracts have lately received attention as bioactive substances for IBD. The ethanol extract from *Haliotis discus hannai* Ino remarkably decreased mucosal tissue damage and lowered the expressions of IL-4, IFN-γ, STAT1, and STAT6 in a mouse model of colitis[83]. Green algae extract also exhibits strong remedial effects against DSS-induced colitis[84].

Phytochemicals

Phytochemicals perform important bioactive functions against oxidative and inflammatory disorders. Plant-

Table 3 Role of natural extracts and phytochemicals against inflammatory bowel disease

Base material	Main compound/agent	Mode of action	Ref.
Extracts			
Mushroom	*Coriolus versicolor* extract	Reduced TNF-α, IL-1β and IL-6	[68]
		Reduced STAT1 and STAT6	
	Cordiceps militaris extract	Decreased epithelial damage	[70]
		Suppressed iNOS and TNF-α mRNA expression	
	Inonotus obliquus extract	Suppressed TNF-α, COX-2, and IFN-γ	[73]
	Ganoderma lucidum extract	Inhibited MAPK phosphorylation and NF-κB activation	[74]
		Decreased histological score	
Fruit extracts			
Prunus mume	*Prunus mume* extract	Suppressed mucosal damage, TNF-α, and iNOS expressions	[75]
Pomegranate	Pomegranate extract (ellagitannins and ellagic acid)	Decreased the expression of TNF-α, COX-2, IL-4, and STAT6	[77]
		Prevented the translocation of NF-κB	[78]
Cranberry	Cranberry fruit/extract	Modulated NF-κB and IL-1β signaling	[79]
	Blueberry extract	Attenuated colon shortening	
		Suppressed pro-inflammatory cytokines	[80]
Averrhoa bilimbi	*Averrhoa bilimbi* L. extract	Prevented oxidation	
		Inhibited pro-inflammatory mediators	[81]
Aronia melanocarpa	*Arronia melanocarpa* juice	Reduced NF-κB translocation	
Ginger	Ginger extract (zingerone)	Decreased mucosal injury	[82]
Marine food		Decrease the level of pro-inflammatory cytokines	[83]
	Haliotis discus hannai Ino extract	Improved colonic damage	[84]
		Decreased TBARS concentration	
		Suppressed NF-κB and IL-1β	
	Green algae extract	Suppressed colonic tissue damage	
		Downregulated IFN-γ and IL-4	
		Ameliorated colonic tissue damage	
		Decreased pro-inflammatory cytokines	
Phytochemicals	Apple polyphenols	Reduced COX-2 and TNF-α	[85]
		Recovered transglutaminase protein	
	Resveratrol	Suppressed NF-κB and TNF-α	[86]
		Reduced clinical score	
	Cardamonin	Reduced histopathological damage	[18]
		Reduced iNOS, NF-κB, TNF-α, COX-2, and caspase-3	
		Suppressed IL-1β and TNF-α	
	Ginsenoside Rg1	Reduced colonic damage and DAI	[90]
		Improved colon shortening and DAI	
	Sulforaphane	Suppressed STAT3 expression	[91]
	Curcumin	Reduced TNF-α, IL-1β, and MPO	[92]
		Attenuated morphological damage	

TLR: Toll-like receptor; TNF-α: Tumor necrosis factor α; IFN-γ: Interferon γ; IL: Interleukin; NF-κB: Nuclear factor κB.

derived bioactive compounds can repress inflammation by inhibiting oxidative damage and interacting with the immune system. In a previous study, apple polyphenol extract reduced mucosal inflammation by reversing transglutaminase depletion in a TNBS-induced colitis rat model[85]. Resveratrol is an important polyphenol found abundantly in peanut, berries, and red grapes. This polyphenol exhibits versatile biological functions that are generally attributed to its modulating actions against oxidative processes and inflammatory pathways[86]. A randomized controlled trial conducted by Samsami-Kor *et al*[87] revealed that patients with UC supplemented with resveratrol had lower inflammation and decreased levels of TNF-α and NF-κB compared with the placebo group. A component from Chinese traditional medicine

called cardamonin was administered to rats with acetic acid-induced colitis, and at the end of the trial, the rats had a reduced DAI score and improved histopathological conditions[18]. Cardamonin supplementation also reduced MDA and MPO activities, and NF-κB, TNF-α, and COX-2 expression. Previous reports suggest that NLRP12, a NOD-like receptor, can attenuate colonic inflammation by downregulating inflammatory cytokines and promoting the growth of useful bacteria in the gut[88,89]. Zhu *et al*[90] reported that Ginsenoside Rg1, a red ginseng compound, inhibited the inflammatory response and colonic damage by upregulating NLRP12 in mice with UC. A broccoli-derived isothiocyanate compound sulforaphane, exhibited anti-colitic activities by preventing colonic atrophy and increasing the expression of the Nrf2-dependent gene in

mice[91]. Curcumin, which is isolated from turmeric, has medicinal application in some Eastern Asian countries, and it is one of the most studied phytochemicals against ulcerative colitis. A study conducted on colitic mice found that curcumin supplementation could improve the histopathological score in the colon by suppressing the activity and the DNA-binding ability of STAT3, and by reducing TNF-α and IL-1β expression[92]. A combined therapy with curcumin, green tea polyphenol, and selenium exhibited outstanding results with decreased inflammatory symptoms and DAI both in human subjects with colitis and in DSS- and TNBS-induced colitic mice[93].

Fatty acids

Polyunsaturated fatty acids (PUFAs) are important pharmaconutrients that can exert therapeutic functions to control inflammatory disorders by modulating the immune response. Therapeutic effects of PUFAs against IBD have been demonstrated over the years, and growing evidence suggests that supplementing with PUFAs through the diet could be an interesting strategy for managing IBD[94]. The role of omega-3 fatty acids, including EPA and DHA, has been investigated in rats. The results indicate that EPA and DHA combined with olive oil and quercitrin reduced the levels of iNOS, COX-2, TNF-α, LTB$_4$, and IL-1β in colitic rats[95]. It is assumed that EPA and DHA-derived metabolites, namely, protectin, resolvin, and maresin are the factors responsible for the anti-inflammatory functions[96]. An adjunct therapy of omega-3 PUFAs with to 5-ASA showed that the dual therapy was more effective in downregulating NF-κB and inducing PPARγ in a rat model of colitis than a higher concentration of 5-ASA alone[97]. Administering EPA together with arachidonic acid (AA) to colitic mice resulted in decreased TNF-α and IL-6 and increased PPARγ[98]. The protective role of conjugated linoleic acid (CLA) on IBD was investigated by Bassaganya-Riera and Hontecillas (2006), and they concluded that CLA could efficiently delay the onset of colitis and decrease the severity of inflammation by influencing PPARγ expression[99]. Alpha linoleic acid from sage oil significantly lowered the inflammatory damage in experimental colitis by decreasing the levels of IL-6, COX-2, and TNF-α[100].

Short chain fatty acids (SCFAs) including acetate, propionate, and butyrate have exhibited therapeutic benefits for colitis. Butyrate limits the immune response and modulates the inflammatory mediators to alleviate mucosal inflammation[101]. In a previous study, butyrate supplementation to colitic rats maintained the integrity of the colonic mucosa by enhancing the production of regulatory T cells (Tregs) in blood and the plasma levels of IL-10 and IL-12[102]. Segain *et al*[103] reported that butyrate inhibited the NF-κB activation and degraded IκBα level in a rat model of colitis. Other SCFAs, including acetate and propionate also showed preventive activity against IBD in mice by inhibiting the expression of immune-related genes and inflammatory mediators, such as NF-κB and IL-6[104].

Bioactive peptides

Dietary peptides have displayed bioactive functions against several illnesses, including chronic inflammation, diabetes, hypertension, and oxidation[105]. Therefore bioactive peptides have the potential to be used as an alternative therapy for IBD and other chronic inflammatory disorders. According to Hou *et al*[106], treatment with alanyl-glutamine in a mouse model of colitis suppressed Th-17 cytokines and macrophage migration to the peritoneal cavity, indicating a reduction in the inflammatory response. Propionyl-L-carnitine, an essential factor of transporting fatty acids in mitochondria reduced mucosal inflammation through antioxidative effects in TNBS-induced colitis[107]. Bovine glycomacropeptide resulted in decreased mucosal damage in the colon, decreased MPO activity, and increased IL-10 in lymphocyte-driven colitis[108]. In a study by Azuma *et al*[109], fish scale gelatin peptide demonstrated anti-inflammatory functions in ulcerative colitis through its inhibitory actions against the activation of NF-κB and the accumulation of monocyte chemotactic protein-1 (MCP-1) in serum. Bioactive peptides isolated from salmon also showed anti-inflammatory functions in experimental colitis in mice[110].

FUTURE TRENDS

The inefficiency of current drug therapies along with the increasing prevalence of IBD from the West towards East Asian and other westernized countries and its recent globalization have triggered a significant amount of research aiming to develop alternative therapies based on natural substances that are highly effective and safe. A coordinated effort based on identifying and solving the environmental and dietary risk factors for IBD will be a priority in the future[111]. As diet is one of the key etiological factors of the disease, a multifaceted dietary intervention involving the elimination of certain foods and the inclusion of food components that can target the underlying causes of IBD is immensely needed. Manipulation of the gut ecosystem with probiotic bacteria is an interesting topic of research in the management of IBD. However, current data for the recommendation of probiotics for chronic metabolic illnesses are still insufficient. Recently, "designer probiotics" has drawn attention as an innovative approach, where genetically engineered bacteria with specific functionalities are administered to patients[112]. Promising results were found when recombinant *Bifidobacteria were* used as carriers for alpha-melanocyte and manganese superoxide dismutase in experimental colitis[113,114]. In recent years, "specific targeting" which allows nutrients or bioactive compounds to reach and target the specific site of inflammation to exert their effects, has become a trending topic of research for IBD control and will

be of tremendous importance in future research[115]. Development of novel cell models that can simulate the GI tract is considered to be a futuristic model of research in the quest for natural alternative therapies for IBD[116]. More importantly, the complete and precise understanding of the pathogenic mechanisms of IBD is necessary, as it will help researchers find suitable and efficacious treatments for IBD using available and prospective natural therapeutic agents. Therefore, future research studies will be centered upon the development of more effective therapeutic strategies for IBD based on health functional materials that will be capable of reaching the target site and exerting their functions to control the underlying pathogenic mechanisms of IBD.

CONCLUSION

Diets and functional foods have emerged as promising alternatives for the prevention and treatment of IBD during the past decade. While diets and dietary habits are key modulating factors involved in the pathogenesis of IBD, several food components such as dietary fibers, probiotics, non-starch polysaccharides, and fat soluble vitamins, have been effective in ameliorating gastrointestinal inflammation. Functional foods and bioactive compounds, including plant-derived extracts, phytochemicals, antioxidants, omega-3 fatty acids, and dietary peptides, have exhibited strong anti-inflammatory effects against IBD both in animal models and human subjects. Functional foods can modulate inflammatory cytokines and can interact with the immune system to produce anti-inflammatory functions against IBD. Therefore, diets and functional foods will play a significant role to control IBD in near future. At the same time, regular food intake, well-managed lifestyle, rest, and medication would require enough attention for the efficient management of IBD.

ACKNOWLEDGMENTS

REFERENCES

1   **Pithadia AB**, Jain S. Treatment of inflammatory bowel disease (IBD). *Pharmacol Rep* 2011; **63**: 629-642 [PMID: 21857074]

2   **Burisch J**, Jess T, Martinato M, Lakatos PL; ECCO -EpiCom. The burden of inflammatory bowel disease in Europe. *J Crohns Colitis* 2013; **7**: 322-337 [PMID: 23395397 DOI: 10.1016/j.crohns.2013.01.010]

3   **Bhandari S**, Larson ME, Kumar N, Stein D. Association of Inflammatory Bowel Disease (IBD) with Depressive Symptoms in the United States Population and Independent Predictors of Depressive Symptoms in an IBD Population: A NHANES Study. *Gut Liver* 2017; **11**: 512-519 [PMID: 28395506 DOI: 10.5009/

gnl16347]

4   **Ng SC**. Epidemiology of inflammatory bowel disease: focus on Asia. *Best Pract Res Clin Gastroenterol* 2014; **28**: 363-372 [PMID: 24913377 DOI: 10.1016/j.bpg.2014.04.003]

5   **Uranga JA**, López-Miranda V, Lombó F, Abalo R. Food, nutrients and nutraceuticals affecting the course of inflammatory bowel disease. *Pharmacol Rep* 2016; **68**: 816-826 [PMID: 27267792 DOI: 10.1016/j.pharep.2016.05.002]

6   **Hur SJ**, Kang SH, Jung HS, Kim SC, Jeon HS, Kim IH, Lee JD. Review of natural products actions on cytokines in inflammatory bowel disease. *Nutr Res* 2012; **32**: 801-816 [PMID: 23176791 DOI: 10.1016/j.nutres.2012.09.013]

7   **Durchschein F**, Petritsch W, Hammer HF. Diet therapy for inflammatory bowel diseases: The established and the new. *World J Gastroenterol* 2016; **22**: 2179-2194 [PMID: 26900283 DOI: 10.3748/wjg.v22.i7.2179]

8   **Paturi G**, Mandimika T, Butts CA, Zhu S, Roy NC, McNabb WC, Ansell J. Influence of dietary blueberry and broccoli on cecal microbiota activity and colon morphology in mdr1a(-/-) mice, a model of inflammatory bowel diseases. *Nutrition* 2012; **28**: 324-330 [PMID: 22113065 DOI: 10.1016/j.nut.2011.07.018]

9   **Hamer HM**, Jonkers DM, Vanhoutvin SA, Troost FJ, Rijkers G, de Bruïne A, Bast A, Venema K, Brummer RJ. Effect of butyrate enemas on inflammation and antioxidant status in the colonic mucosa of patients with ulcerative colitis in remission. *Clin Nutr* 2010; **29**: 738-744 [PMID: 20471725 DOI: 10.1016/j.clnu.2010.04.002]

10 **Le Leu RK**, Young GP, Hu Y, Winter J, Conlon MA. Dietary red meat aggravates dextran sulfate sodium-induced colitis in mice whereas resistant starch attenuates inflammation. *Dig Dis Sci* 2013; **58**: 3475-3482 [PMID: 23990000 DOI: 10.1007/s10620-013-2844-1]

11 **Di Luccia B**, Manzo N, Baccigalupi L, Calabrò V, Crescenzi E, Ricca E, Pollice A. Lactobacillus gasseri SF1183 affects intestinal epithelial cell survival and growth. *PLoS One* 2013; **8**: e69102 [PMID: 23894414 DOI: 10.1016/j.jff.2017.12.049]

12 **Lim HS**, Kim SK, Hong SJ. Food Elimination Diet and Nutritional Deficiency in Patients with Inflammatory Bowel Disease. *Clin Nutr Res* 2018; **7**: 48-55 [PMID: 29423389 DOI: 10.7762/cnr.2018.7.1.48]

13 **Farombi EO**, Adedara IA, Ajayi BO, Ayepola OR, Egbeme EE. Kolaviron, a natural antioxidant and anti-inflammatory phytochemical prevents dextran sulphate sodium-induced colitis in rats. *Basic Clin Pharmacol Toxicol* 2013; **113**: 49-55 [PMID: 23336970 DOI: 10.1111/bcpt.12050]

14 **Sobczak M**, Zakrzewski PK, Cygankiewicz AI, Mokrowiecka A, Chen C, Sałaga M, Małecka-Panas E, Kordek R, Krajewska WM, Fichna J. Anti-inflammatory action of a novel orally available peptide 317 in mouse models of inflammatory bowel diseases. *Pharmacol Rep* 2014; **66**: 741-750 [PMID: 25149976 DOI: 10.1016/j.pharep.2014.03.007]

15 **Chen P**, Zhou X, Zhang L, Shan M, Bao B, Cao Y, Kang A, Ding A. Anti-inflammatory effects of Huangqin tang extract in mice on ulcerative colitis. *J Ethnopharmacol* 2015; **162**: 207-214 [PMID: 25576893 DOI: 10.1016/j.jep.20z14.12.039]

16 **Zhang H**, Liu R, Tsao R. Anthocyanin-rich phenolic extracts of purple root vegetables inhibit pro-inflammatory cytokines induced by H2O2 and enhance antioxidant enzyme activities in Caco-2 cells. *J Funct Foods* 2016; **22**: 363-375 [DOI: 10.1016/j.jff.2016.01.004]

17 **Hwang YH**, Kim DG, Li W, Yang HJ, Yim NH, Ma JY. Anti-inflammatory effects of Forsythia suspensa in dextran sulfate sodium-induced colitis. *J Ethnopharmacol* 2017; **206**: 73-77 [PMID: 28502906 DOI: 10.1016/j.jep.2017.05.011]

18 **Ali AA**, Abd Al Haleem EN, Khaleel SA, Sallam AS. Protective effect of cardamonin against acetic acid-induced ulcerative colitis in rats. *Pharmacol Rep* 2017; **69**: 268-275 [PMID: 28129600 DOI: 10.1016/j.pharep.2016.11.002]

19 **Kirsner JB**. Historical origins of current IBD concepts. *World J Gastroenterol* 2001; **7**: 175-184 [PMID: 11819757 DOI: 10.3748/

wjg.v7.i2.175]

20   **Knight-Sepulveda K**, Kais S, Santaolalla R, Abreu MT. Diet and Inflammatory Bowel Disease. *Gastroenterol Hepatol* (NY) 2015; **11**: 511-520 [PMID: 27118948]

21   **Ardizzone S**, Bianchi Porro G. Biologic therapy for inflammatory bowel disease. *Drugs* 2005; **65**: 2253-2286 [PMID: 16266194 DOI: 10.2165/00003495-200565160-00002]

22   **Zhao L**, Wu H, Zhao A, Lu H, Sun W, Ma C, Yang Y, Xin X, Zou H, Qiu M, Jia W. The in vivo and in vitro study of polysaccharides from a two-herb formula on ulcerative colitis and potential mechanism of action. *J Ethnopharmacol* 2014; **153**: 151-159 [PMID: 24548752 DOI: 10.1016/j.jep.2014.02.008]

23   **Horváth K**, Varga C, Berkó A, Pósa A, László F, Whittle BJ. The involvement of heme oxygenase-1 activity in the therapeutic actions of 5-aminosalicylic acid in rat colitis. *Eur J Pharmacol* 2008; **581**: 315-323 [PMID: 18215658 DOI: 10.1016/j.ejphar.2007.12.004]

24   **Ito K**, Chung KF, Adcock IM. Update on glucocorticoid action and resistance. *J Allergy Clin Immunol* 2006; **117**: 522-543 [PMID: 16522450 DOI: 10.1016/j.jaci.2006.01.032]

25   **Renna S**, Cottone M, Orlando A. Optimization of the treatment with immunosuppressants and biologics in inflammatory bowel disease. *World J Gastroenterol* 2014; **20**: 9675-9690 [PMID: 25110407 DOI: 10.3748/wjg.v20.i29.9675]

26   **Nitzan O**, Elias M, Peretz A, Saliba W. Role of antibiotics for treatment of inflammatory bowel disease. *World J Gastroenterol* 2016; **22**: 1078-1087 [PMID: 26811648 DOI: 10.3748/wjg.v22.i3.1078]

27   **Crowe JS**, Roberts KJ, Carlton TM, Maggiore L, Cubitt MF, Clare S, Harcourt K, Reckless J, MacDonald TT, Ray KP, Vossenkämper A, West MR. Preclinical Development of a Novel, Orally-Administered Anti-Tumour Necrosis Factor Domain Antibody for the Treatment of Inflammatory Bowel Disease. *Sci Rep* 2018; **8**: 4941 [PMID: 29563546 DOI: 10.1038/s41598-018-23277-7]

28   **Moura FA**, de Andrade KQ, dos Santos JC, Araújo OR, Goulart MO. Antioxidant therapy for treatment of inflammatory bowel disease: Does it work? *Redox Biol* 2015; **6**: 617-639 [PMID: 26520808 DOI: 10.1016/j.redox.2015.10.006]

29   **Currò D**, Ianiro G, Pecere S, Bibbò S, Cammarota G. Probiotics, fibre and herbal medicinal products for functional and inflammatory bowel disorders. *Br J Pharmacol* 2017; **174**: 1426-1449 [PMID: 27696378 DOI: 10.1111/bph.13632]

30   **Derikx LA**, Dieleman LA, Hoentjen F. Probiotics and prebiotics in ulcerative colitis. *Best Pract Res Clin Gastroenterol* 2016; **30**: 55-71 [PMID: 27048897 DOI: 10.1016/j.bpg.2016.02.005]

31   **Srutkova D**, Schwarzer M, Hudcovic T, Zakostelska Z, Drab V, Spanova A, Rittich B, Kozakova H, Schabussova I. Bifidobacterium longum CCM 7952 Promotes Epithelial Barrier Function and Prevents Acute DSS-Induced Colitis in Strictly Strain-Specific Manner. *PLoS One* 2015; **10**: e0134050 [PMID: 26218526 DOI: 10.1371/journal.pone.0134050]

32   **Lee JH**, Lee B, Lee HS, Bae EA, Lee H, Ahn YT, Lim KS, Huh CS, Kim DH. Lactobacillus suntoryeus inhibits pro-inflammatory cytokine expression and TLR-4-linked NF-kappaB activation in experimental colitis. *Int J Colorectal Dis* 2009; **24**: 231-237 [PMID: 19050899 DOI: 10.1007/s00384-008-0618-6]

33   **Kawahara M**, Nemoto M, Nakata T, Kondo S, Takahashi H, Kimura B, Kuda T. Anti-inflammatory properties of fermented soy milk with Lactococcus lactis subsp. lactis S-SU2 in murine macrophage RAW264.7 cells and DSS-induced IBD model mice. *Int Immunopharmacol* 2015; **26**: 295-303 [PMID: 25887264 DOI: 10.1016/j.intimp.2015.04.004]

34   **Park JS**, Joe I, Rhee PD, Jeong CS, Jeong G. A lactic acid bacterium isolated from kimchi ameliorates intestinal inflammation in DSS-induced colitis. *J Microbiol* 2017; **55**: 304-310 [PMID: 28124779 DOI: 10.1007/s12275-017-6447-y]

35   **Shigemori S**, Watanabe T, Kudoh K, Ihara M, Nigar S, Yamamoto Y, Suda Y, Sato T, Kitazawa H, Shimosato T. Oral delivery of Lactococcus lactis that secretes bioactive heme oxygenase-1 alleviates development of acute colitis in mice.

36   **Yokota Y**, Shikano A, Kuda T, Takei M, Takahashi H, Kimura B. Lactobacillus plantarum AN1 cells increase caecal L. reuteri in an ICR mouse model of dextran sodium sulphate-induced inflammatory bowel disease. *Int Immunopharmacol* 2018; **56**: 119-127 [PMID: 29414641 DOI: 10.1016/j.intimp.2018.01.020]

37   **Le B**, Yang SH. Efficacy of Lactobacillus plantarum in prevention of inflammatory bowel disease. *Toxicol Rep* 2018; **5**: 314-317 [DOI: 10.1016/j.toxrep.2018.02.007]

38   **Seo S**, Shin J-S, Lee WS, Rhee YK, Cho CW, Hong H-D, Lee K-T. Anti-colitis effect of Lactobacillus sakei K040706 via suppression of inflammatory responses in the dextran sulfate sodium-induced colitis mice model. *J Funct Food* 2017; **29**: 256-268 [DOI: 10.1016/j.jff.2016.12.045]

39   **Gholami M**, Ghasemi-Niri SF, Maqbool F, Baeeri M, Memariani Z, Pousti I, Abdollahi M. Experimental and Pathalogical study of Pistacia atlantica, butyrate, Lactobacillus casei and their combination on rat ulcerative colitis model. *Pathol Res Pract* 2016; **212**: 500-508 [PMID: 26972417 DOI: 10.1016/j.prp.2016.02.024]

40   **Lim SM**, Kim DH. Bifidobacterium adolescentis IM38 ameliorates high-fat diet-induced colitis in mice by inhibiting NF-κB activation and lipopolysaccharide production by gut microbiota. *Nutr Res* 2017; **41**: 86-96 [PMID: 28479226 DOI: 10.1016/j.nutres.2017.04.003]

41   **Satish Kumar CS**, Kondal Reddy K, Boobalan G, Gopala Reddy A, Sudha Rani Chowdhary CH, Vinoth A, Jayakanth K, Srinivasa Rao G. Immunomodulatory effects of Bifidobacterium bifidum 231 on trinitrobenzenesulfonic acid-induced ulcerative colitis in rats. *Res Vet Sci* 2017; **110**: 40-46 [PMID: 28159236 DOI: 10.1016/j.rvsc.2016.10.010]

42   **Liu X**, Wu Y, Li F, Zhang D. Dietary fiber intake reduces risk of inflammatory bowel disease: result from a meta-analysis. *Nutr Res* 2015; **35**: 753-758 [PMID: 26126709 DOI: 10.1016/j.nutres.2015.05.021]

43   **Nie Y**, Lin Q, Luo F. Effects of Non-Starch Polysaccharides on Inflammatory Bowel Disease. *Int J Mol Sci* 2017; **18**: pii: E1372 [PMID: 28654020 DOI: 10.3390/ijms18071372]

44   **Suwannaporn P**, Thepwong K, Tester R, Al-Ghazzewi F, Piggott J, Shen N, Chen Z Chen F, Yang J, Zhang D, Tang M. Tolerance and nutritional therapy of dietary fibre from konjac glucomannan hydrolysates for patients with inflammatory bowel disease (IBD). *Bioact Carbohydr Diet Fiber* 2013; **2**: 93-98 [DOI: 10.1016/j.bcdf.2013.09.005]

45   **Asano T**, Tanaka K, Suemasu S, Ishihara T, Tahara K, Suzuki T, Suzuki H, Fukudo S, Mizushima T. Effects of β-(1,3-1,6)-D-glucan on irritable bowel syndrome-related colonic hypersensitivity. *Biochem Biophys Res Commun* 2012; **420**: 444-449 [PMID: 22430139 DOI: 10.1016/j.bbrc.2012.03.015]

46   **Liu B**, Lin Q, Yang T, Zeng L, Shi L, Chen Y, Luo F. Oat β-glucan ameliorates dextran sulfate sodium (DSS)-induced ulcerative colitis in mice. *Food Funct* 2015; **6**: 3454-3463 [PMID: 26292622 DOI: 10.1039/c5fo00563a]

47   **Lavi I**, Levinson D, Peri I, Nimri L, Hadar Y, Schwartz B. Orally administered glucans from the edible mushroom Pleurotus pulmonarius reduce acute inflammation in dextran sulfate sodium-induced experimental colitis. *Br J Nutr* 2010; **103**: 393-402 [PMID: 19772681 DOI: 10.1017/S0007114509991760]

48   **Lee KH**, Park M, Ji KY, Lee HY, Jang JH, Yoon IJ, Oh SS, Kim SM, Jeong YH, Yun CH, Kim MK, Lee IY, Choi HR, Ko KS, Kang HS. Bacterial β-(1,3)-glucan prevents DSS-induced IBD by restoring the reduced population of regulatory T cells. *Immunobiology* 2014; **219**: 802-812 [PMID: 25092569 DOI: 10.1016/j.imbio.2014.07.003]

49   **Azuma K**, Osaki T, Ifuku F, Saimoto H, Morimoto M, Takashima O, Tsuka T, Imagawa T, Okamoto Y, Minami S. Suppressive effects of cellulose nanofibers—made from adlay and seaweed—on colon inflammation in an inflammatory bowel-disease model. *Bioact Carbohydr Diet Fiber* 2013; **3**: 65-72 [DOI: 10.1016/j.bcdf.2013.09.006]

Microb Cell Fact 2015; **14**: 189 [PMID: 26608030 DOI: 10.1186/s12934-015-0378-2]

50   **Azuma K**, Osaki T, Ifuku F, Saimoto H, Morimoto M, Takashima O, Tsuka T, Imagawa T, Okamoto Y, Minami S. Anti-inflammatory effects of cellulose nanofiber made from pear in inflammatory bowel disease model. *Bioact Carbohydr Diet Fiber* 2014; **3**: 1-10 [DOI: 10.1016/j.bcdf.2013.11.001]

51   **Capitán-Cañadas F**, Ocón B, Aranda CJ, Anzola A, Suárez MD, Zarzuelo A, de Medina FS, Martínez-Augustin O. Fructooligosaccharides exert intestinal anti-inflammatory activity in the CD4+ CD62L+ T cell transfer model of colitis in C57BL/6J mice. *Eur J Nutr* 2016; **55**: 1445-1454 [PMID: 26154776 DOI: 10.1007/s00394-015-0962-6]

52   **Lara-Villoslada F**, Debras E, Nieto A, Concha A, Gálvez J, López-Huertas E, Boza J, Obled C, Xaus J. Oligosaccharides isolated from goat milk reduce intestinal inflammation in a rat model of dextran sulfate-induced colitis. *Clin Nutr* 2006; **25**: 477-488 [PMID: 16375993 DOI: 10.1016/j.clnu.2005.11.004]

53   **Štofilová J**, Szabadosová V, Hrčková G, Salaj R, Bertková I, Hijová E, Strojný L, Bomba A. Co-administration of a probiotic strain Lactobacillus plantarum LS/07 CCM7766 with prebiotic inulin alleviates the intestinal inflammation in rats exposed to N,N-dimethylhydrazine. *Int Immunopharmacol* 2015; **24**: 361-368 [PMID: 25536541 DOI: 10.1016/j.intimp.2014.12.022]

54   **Gubatan J**, Mitsuhashi S, Zenlea T, Rosenberg L, Robson S, Moss AC. Low Serum Vitamin D During Remission Increases Risk of Clinical Relapse in Patients With Ulcerative Colitis. *Clin Gastroenterol Hepatol* 2017; **15**: 240-246.e1 [PMID: 27266980 DOI: 10.1016/j.cgh.2016.05.035]

55   **Meckel K**, Li YC, Lim J, Kocherginsky M, Weber C, Almoghrabi A, Chen X, Kaboff A, Sadiq F, Hanauer SB, Cohen RD, Kwon J, Rubin DT, Hanan I, Sakuraba A, Yen E, Bissonnette M, Pekow J. Serum 25-hydroxyvitamin D concentration is inversely associated with mucosal inflammation in patients with ulcerative colitis. *Am J Clin Nutr* 2016; **104**: 113-120 [PMID: 27281309 DOI: 10.3945/ajcn.115.123786]

56   **Chen SW**, Wang PY, Zhu J, Chen GW, Zhang JL, Chen ZY, Zuo S, Liu YC, Pan YS. Protective effect of 1,25-dihydroxyvitamin d3 on lipopolysaccharide-induced intestinal epithelial tight junction injury in caco-2 cell monolayers. *Inflammation* 2015; **38**: 375-383 [PMID: 25344656 DOI: 10.1007/s10753-014-0041-9]

57   **Zhu Y**, Mahon BD, Froicu M, Cantorna MT. Calcium and 1 alpha,25-dihydroxyvitamin D3 target the TNF-alpha pathway to suppress experimental inflammatory bowel disease. *Eur J Immunol* 2005; **35**: 217-224 [PMID: 15593122 DOI: 10.1002/eji.200425491]

58   **Bartels LE**, Jørgensen SP, Agnholt J, Kelsen J, Hvas CL, Dahlerup JF. 1,25-dihydroxyvitamin D3 and dexamethasone increase interleukin-10 production in CD4+ T cells from patients with Crohn's disease. *Int Immunopharmacol* 2007; **7**: 1755-1764 [PMID: 17996686 DOI: 10.1016/j.intimp.2007.09.016]

59   **Bendix-Struve M**, Bartels LE, Agnholt J, Dige A, Jørgensen SP, Dahlerup JF. Vitamin D3 treatment of Crohn's disease patients increases stimulated T cell IL-6 production and proliferation. *Aliment Pharmacol Ther* 2010; **32**: 1364-1372 [PMID: 21050239 DOI: 10.1111/j.1365-2036.2010.04463.x]

60   **Reifen R**, Levy E, Berkovich Z, Tirosh O. Vitamin A exerts its antiinflammatory activities in colitis through preservation of mitochondrial activity. *Nutrition* 2015; **31**: 1402-1407 [PMID: 26429662 DOI: 10.1016/j.nut.2015.05.011]

61   **Gottschall E**. Breaking the Vicious Cycle: Intestinal Health Through Diet. Balti¬more, Canada: Kirkton Press; 2012

62   **Cohen SA**, Gold BD, Oliva S, Lewis J, Stallworth A, Koch B, Eshee L, Mason D. Clinical and mucosal improvement with specific carbohydrate diet in pediatric Crohn disease. *J Pediatr Gastroenterol Nutr* 2014; **59**: 516-521 [PMID: 24897165 DOI: 10.1097/MPG.0000000000000449]

63   **Marion-Letellier R**, Savoye G, Ghosh S. IBD: In Food We Trust. *J Crohns Colitis* 2016; **10**: 1351-1361 [PMID: 27194533 DOI: 10.1093/ecco-jcc/jjw106]

64   **Marcason W**. What is the FODMAP diet? *J Acad Nutr Diet* 2012; **112**: 1696 [PMID: 23017576]

65   **Gearry RB**, Irving PM, Barrett JS, Nathan DM, Shepherd SJ, Gibson PR. Reduction of dietary poorly absorbed short-chain carbohydrates (FODMAPs) improves abdominal symptoms in patients with inflammatory bowel disease-a pilot study. *J Crohns Colitis* 2009; **3**: 8-14 [PMID: 21172242 DOI: 10.1016/j.crohns.2008.09.004]

66   **Pedersen N**, Ankersen DV, Felding M, Wachmann H, Végh Z, Molzen L, Burisch J, Andersen JR, Munkholm P. Low-FODMAP diet reduces irritable bowel symptoms in patients with inflammatory bowel disease. *World J Gastroenterol* 2017; **23**: 3356-3366 [PMID: 28566897 DOI: 10.3748/wjg.v23.i18.3356]

67   **Larussa T**, Imeneo M, Luzza F. Potential role of nutraceutical compounds in inflammatory bowel disease. *World J Gastroenterol* 2017; **23**: 2483-2492 [PMID: 28465632 DOI: 10.3748/wjg.v23.i14.2483]

68   **Lim BO**. Coriolus versicolor suppresses inflammatory bowel disease by Inhibiting the expression of STAT1 and STAT6 associated with IFN-γ and IL-4 expression. *Phytother Res* 2011; **25**: 1257-1261 [PMID: 21796702 DOI: 10.1002/ptr.3378]

69   **Won SY**, Park EH. Anti-inflammatory and related pharmacological activities of cultured mycelia and fruiting bodies of Cordyceps militaris. *J Ethnopharmacol* 2005; **96**: 555-561 [PMID: 15619578 DOI: 10.1016/j.jep.2004.10.009]

70   **Han ES**, Oh JY, Park HJ. Cordyceps militaris extract suppresses dextran sodium sulfate-induced acute colitis in mice and production of inflammatory mediators from macrophages and mast cells. *J Ethnopharmacol* 2011; **134**: 703-710 [PMID: 21277968 DOI: 10.1016/j.jep.2011.01.022]

71   **Jeon TI**, Hwang SG, Lim BO, Park DK. Extracts of Phellinus linteus grown on germinated brown rice suppress liver damage induced by carbon tetrachloride in rats. *Biotechnol Lett* 2003; **25**: 2093-2096 [PMID: 14969415]

72   **Debnath T**, Park SR, Kim DH, Jo JE, Lim BO. Anti-oxidant and anti-inflammatory activities of Inonotus obliquus and germinated brown rice extracts. *Molecules* 2013; **18**: 9293-9304 [PMID: 23917116 DOI: 10.3390/molecules18089293]

73   **Debnath T**, Hasnat MA, Pervin M, Lee SY, Park SR, Kim DH, Kweon HJ, Kim JM, Lim BO. Chaga Mushroom (Inonotus obliquus) Grown on Germinated Brown Rice Suppresses Inflammation Associated with Colitis in Mice. *Food Sci Biotechnol* 2012; **21**: 1235-1241 [DOI: 10.1007/s10068-012-0162-6]

74   **Hasnat MA**, Pervin M, Cha KM, Kim SK, Lim BO. Anti-inflammatory activity on mice of extract of Ganoderma lucidum grown on rice via modulation of MAPK and NF-κB pathways. *Phytochemistry* 2015; **114**: 125-136 [PMID: 25457483 DOI: 10.1016/j.phytochem.2014.10.019]

75   **Jin HL**, Lee BR, Lim KJ, Debnath T, Shin HM, Lim BO. Anti-Inflammatory Effects of Prunus mume Mixture in Colitis Induced by Dextran Sodium Sulfate. *Kor J Medicinal Crop Sci* 2011; **19**: 16-23 [DOI: 10.7783/KJMCS.2011.19.1.016]

76   **Kamali M**, Tavakoli H, Khodadoost M, Daghaghzadeh H, Kamalinejad M, Gachkar L, Mansourian M, Adibi P. Efficacy of the Punica granatum peels aqueous extract for symptom management in ulcerative colitis patients. A randomized, placebo-controlled, clinical trial. *Complement Ther Clin Pract* 2015; **21**: 141-146 [PMID: 26256131 DOI: 10.1016/j.ctcp.2015.03.001]

77   **Rosillo MA**, Sánchez-Hidalgo M, Cárdeno A, Aparicio-Soto M, Sánchez-Fidalgo S, Villegas I, de la Lastra CA. Dietary supplementation of an ellagic acid-enriched pomegranate extract attenuates chronic colonic inflammation in rats. *Pharmacol Res* 2012; **66**: 235-242 [PMID: 22677088 DOI: 10.1016/j.phrs.2012.05.006]

78   **Xiao X**, Kim J, Sun Q, Kim D, Park CS, Lu TS, Park Y. Preventive effects of cranberry products on experimental colitis induced by dextran sulphate sodium in mice. *Food Chem* 2015; **167**: 438-446 [PMID: 25149009 DOI: 10.1016/j.foodchem.2014.07.006]

79   **Pervin M**, Hasnat MA, Lim JH, Lee YM, Kim EO, Um BH, Lim BO. Preventive and therapeutic effects of blueberry (Vaccinium corymbosum) extract against DSS-induced ulcerative colitis by regulation of antioxidant and inflammatory mediators. *J Nutr Biochem* 2016; **28**: 103-113 [PMID: 26878787 DOI: 10.1016/

j.jnutbio.2015.10.006]

80   **Suluvoy JK**, Sakthivel KM, Guruvayoorappan C, Berlin Grace VM. Protective effect of Averrhoa bilimbi L. fruit extract on ulcerative colitis in wistar rats via regulation of inflammatory mediators and cytokines. *Biomed Pharmacother* 2017; **91**: 1113-1121 [PMID: 28531922 DOI: 10.1016/j.biopha.2017.05.057]

81   **Valcheva-Kuzmanova S**, Kuzmanov A, Kuzmanova V, Tzaneva M. Aronia melanocarpa fruit juice ameliorates the symptoms of inflammatory bowel disease in TNBS-induced colitis in rats. *Food Chem Toxicol* 2018; **113**: 33-39 [PMID: 29331733 DOI: 10.1016/j.fct.2018.01.011]

82   **Hsiang CY**, Lo HY, Huang HC, Li CC, Wu SL, Ho TY. Ginger extract and zingerone ameliorated trinitrobenzene sulphonic acid-induced colitis in mice via modulation of nuclear factor-κB activity and interleukin-1β signalling pathway. *Food Chem* 2013; **136**: 170-177 [PMID: 23017409 DOI: 10.1016/j.foodchem.2012.07.124]

83   **Debnath T**, Mijan MA, Kim DH, Jo JE, Kim YO, Lee JJ, Han JP, Lim BO. Anti-inflammatory effects of Haliotis discus hannai Ino on dextran sulfate sodium-induced colitis in mice. *J Food Biochem* 2015; **39**: 209-217 [DOI: 10.1111/jfbc.12118]

84   **Bitencourt MA**, Silva HM, Abílio GM, Miranda GE, Moura AM, Araújo-Júnior JX, Silveira EJ, Santos BV, Souto JT. Anti-inflammatory effects of methanolic extract of green algae Caulerpa mexicana in a murine model of ulcerative colitis. *Rev Brasileira Farmacog* 2015; **25**: 677-682 [DOI: 10.1016/j.bjp.2015.10.001]

85   **D'Argenio G**, Mazzone G, Tuccillo C, Ribecco MT, Graziani G, Gravina AG, Caserta S, Guido S, Fogliano V, Caporaso N, Romano M. Apple polyphenols extract (APE) improves colon damage in a rat model of colitis. *Dig Liver Dis* 2012; **44**: 555-562 [PMID: 22381211 DOI: 10.1016/j.dld.2012.01.009]

86   **Martín AR**, Villegas I, Sánchez-Hidalgo M, de la Lastra CA. The effects of resveratrol, a phytoalexin derived from red wines, on chronic inflammation induced in an experimentally induced colitis model. *Br J Pharmacol* 2006; **147**: 873-885 [PMID: 16474422 DOI: 10.1038/sj.bjp.0706469]

87   **Samsami-Kor M**, Daryani NE, Asl PR, Hekmatdoost A. Anti-Inflammatory Effects of Resveratrol in Patients with Ulcerative Colitis: A Randomized, Double-Blind, Placebo-controlled Pilot Study. *Arch Med Res* 2015; **46**: 280-285 [PMID: 26002728 DOI: 10.1016/j.arcmed.2015.05.005]

88   **Chen L**, Wilson JE, Koenigsknecht MJ, Chou WC, Montgomery SA, Truax AD, Brickey WJ, Packey CD, Maharshak N, Matsushima GK, Plevy SE, Young VB, Sartor RB, Ting JP. NLRP12 attenuates colon inflammation by maintaining colonic microbial diversity and promoting protective commensal bacterial growth. *Nat Immunol* 2017; **18**: 541-551 [PMID: 28288099 DOI: 10.1038/ni.3690]

89   **Zaki MH**, Vogel P, Malireddi RK, Body-Malapel M, Anand PK, Bertin J, Green DR, Lamkanfi M, Kanneganti TD. The NOD-like receptor NLRP12 attenuates colon inflammation and tumorigenesis. *Cancer Cell* 2011; **20**: 649-660 [PMID: 22094258 DOI: 10.1016/j.ccr.2011.10.022]

90   **Zhu G**, Wang H, Wang T, Shi F. Ginsenoside Rg1 attenuates the inflammatory response in DSS-induced mice colitis. *Int Immunopharmacol* 2017; **50**: 1-5 [PMID: 28605639 DOI: 10.1016/j.intimp.2017.06.002]

91   **Wagner AE**, Will O, Sturm C, Lipinski S, Rosenstiel P, Rimbach G. DSS-induced acute colitis in C57BL/6 mice is mitigated by sulforaphane pre-treatment. *J Nutr Biochem* 2013; **24**: 2085-2091 [PMID: 24231100 DOI: 10.1016/j.jnutbio.2013.07.009]

92   **Liu L**, Liu YL, Liu GX, Chen X, Yang K, Yang YX, Xie Q, Gan HK, Huang XL, Gan HT. Curcumin ameliorates dextran sulfate sodium-induced experimental colitis by blocking STAT3 signaling pathway. *Int Immunopharmacol* 2013; **17**: 314-320 [PMID: 23856612 DOI: 10.1016/j.intimp.2013.06.020]

93   **Shapira S**, Leshno A, Katz D, Maharshak N, Hevroni G, Jean-David M, Kraus S, Galazan L, Aroch I, Kazanov D, Hallack A, Becker S, Umanski M, Moshkowitz M, Dotan I, Arber N. Of mice and men: a novel dietary supplement for the treatment of ulcerative colitis. *Therap Adv Gastroenterol* 2017; **11**: 1756283X17741864

[PMID: 29383023 DOI: 10.1177/1756283X17741864]

94   **Uchiyama K**, Nakamura M, Odahara S, Koido S, Katahira K, Shiraishi H, Ohkusa T, Fujise K, Tajiri H. N-3 polyunsaturated fatty acid diet therapy for patients with inflammatory bowel disease. *Inflamm Bowel Dis* 2010; **16**: 1696-1707 [PMID: 20222122 DOI: 10.1002/ibd.21251]

95   **Camuesco D**, Comalada M, Concha A, Nieto A, Sierra S, Xaus J, Zarzuelo A, Gálvez J. Intestinal anti-inflammatory activity of combined quercitrin and dietary olive oil supplemented with fish oil, rich in EPA and DHA (n-3) polyunsaturated fatty acids, in rats with DSS-induced colitis. *Clin Nutr* 2006; **25**: 466-476 [PMID: 16698151 DOI: 10.1016/j.clnu.2005.12.009]

96   **Schwanke RC**, Marcon R, Bento AF, Calixto JB. EPA- and DHA-derived resolvins' actions in inflammatory bowel disease. *Eur J Pharmacol* 2016; **785**: 156-164 [PMID: 26325092 DOI: 10.1016/j.ejphar.2015.08.050]

97   **Mbodji K**, Charpentier C, Guérin C, Querec C, Bole-Feysot C, Aziz M, Savoye G, Déchelotte P, Marion-Letellier R. Adjunct therapy of n-3 fatty acids to 5-ASA ameliorates inflammatory score and decreases NF-κB in rats with TNBS-induced colitis. *J Nutr Biochem* 2013; **24**: 700-705 [PMID: 22841543 DOI: 10.1016/j.jnutbio.2012.03.022]

98   **Roy N**, Barnett M, Knoch B, Dommels Y, McNabb W. Nutrigenomics applied to an animal model of Inflammatory Bowel Diseases: transcriptomic analysis of the effects of eicosapentaenoic acid- and arachidonic acid-enriched diets. *Mutat Res* 2007; **622**: 103-116 [PMID: 17574631 DOI: 10.1016/j.mrfmmm.2007.04.003]

99   **Bassaganya-Riera J**, Hontecillas R. CLA and n-3 PUFA differentially modulate clinical activity and colonic PPAR-responsive gene expression in a pig model of experimental IBD. *Clin Nutr* 2006; **25**: 454-465 [PMID: 16698153 DOI: 10.1016/j.clnu.2005.12.008]

100   **Reifen R**, Karlinsky A, Stark AH, Berkovich Z, Nyska A. α-Linolenic acid (ALA) is an anti-inflammatory agent in inflammatory bowel disease. *J Nutr Biochem* 2015; **26**: 1632-1640 [PMID: 26350254 DOI: 10.1016/j.jnutbio.2015.08.006]

101   **Cushing K**, Alvarado DM, Ciorba MA. Butyrate and Mucosal Inflammation: New Scientific Evidence Supports Clinical Observation. *Clin Transl Gastroenterol* 2015; **6**: e108 [PMID: 26312412 DOI: 10.1038/ctg.2015.34]

102   **Zhang M**, Zhou Q, Dorfman RG, Huang X, Fan T, Zhang H, Zhang J, Yu C. Butyrate inhibits interleukin-17 and generates Tregs to ameliorate colorectal colitis in rats. *BMC Gastroenterol* 2016; **16**: 84 [PMID: 27473867 DOI: 10.1186/s12876-016-0500-x]

103   **Segain JP**, Raingeard de la Blétière D, Bourreille A, Leray V, Gervois N, Rosales C, Ferrier L, Bonnet C, Blottière HM, Galmiche JP. Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. *Gut* 2000; **47**: 397-403 [PMID: 10940278]

104   **Tedelind S**, Westberg F, Kjerrulf M, Vidal A. Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: a study with relevance to inflammatory bowel disease. *World J Gastroenterol* 2007; **13**: 2826-2832 [PMID: 17569118 DOI: 10.3748/wjg.v13.i20.2826]

105   **Chakrabarti S**, Jahandideh F, Wu J. Food-derived bioactive peptides on inflammation and oxidative stress. *Biomed Res Int* 2014; **2014**: 608979 [PMID: 24527452 DOI: 10.1155/2014/608979]

106   **Hou YC**, Liu JJ, Pai MH, Tsou SS, Yeh SL. Alanyl-glutamine administration suppresses Th17 and reduces inflammatory reaction in dextran sulfate sodium-induced acute colitis. *Int Immunopharmacol* 2013; **17**: 1-8 [PMID: 23721689 DOI: 10.1016/j.intimp.2013.05.004]

107   **Scioli MG**, Stasi MA, Passeri D, Doldo E, Costanza G, Camerini R, Fociani P, Arcuri G, Lombardo K, Pace S, Borsini F, Orlandi A. Propionyl-L-Carnitine is Efficacious in Ulcerative Colitis Through its Action on the Immune Function and Microvasculature. *Clin Transl Gastroenterol* 2014; **5**: e55 [PMID: 24646507 DOI: 10.1038/ctg.2014.4]

108   **Ortega-González M**, Capitán-Cañadas F, Requena P, Ocón B, Romero-Calvo I, Aranda C, Suárez MD, Zarzuelo A, Sánchez de Medina F, Martínez-Augustin O. Validation of bovine

glycomacropeptide as an intestinal anti-inflammatory nutraceutical in the lymphocyte-transfer model of colitis. *Br J Nutr* 2014; **111**: 1202-1212 [PMID: 24229852 DOI: 10.1017/S0007114513003590]

109   **Azuma K**, Osaki T, Tsuka T, Imagawa T, Okamoto Y, and Minami S. Effects of fish scale collagen peptide on an experimental ulcerative colitis mouse model. *Pharm Nutr* 2014; **2**: 161-168 [DOI: 10.1016/j.phanu.2014.10.001]

110   **Grimstad T**, Bjørndal B, Cacabelos D, Aasprong OG, Omdal R, Svardal A, Bohov P, Pamplona R, Portero-Otin M, Berge RK, Hausken T. A salmon peptide diet alleviates experimental colitis as compared with fish oil. *J Nutr Sci* 2013; **2**: e2 [PMID: 25191568 DOI: 10.1017/jns.2012.23]

111   **Kaplan GG**, Ng SC. Understanding and Preventing the Global Increase of Inflammatory Bowel Disease. *Gastroenterology* 2017; **152**: 313-321.e2 [PMID: 27793607 DOI: 10.1053/j.gastro.2016.10.020]

112   **Singh B**, Mal G, Marotta F. Designer Probiotics: Paving the Way to Living Therapeutics. *Trends Biotechnol* 2017; **35**: 679-682 [PMID: 28483159 DOI: 10.1016/j.tibtech.2017.04.001]

113   **Wei P**, Yang Y, Liu Z, Huang J, Gong Y, Sun H. Oral Bifidobacterium longum expressing alpha-melanocyte-stimulating hormone to fight experimental colitis. *Drug Deliv* 2016; **23**: 2058-2064 [PMID: 26673899 DOI: 10.3109/10717544.2015.1122672]

114   **Liu M**, Li S, Zhang Q, Xu Z, Wang J, Sun H. Oral engineered Bifidobacterium longum expressing rhMnSOD to suppress experimental colitis. *Int Immunopharmacol* 2018; **57**: 25-32 [PMID: 29455070 DOI: 10.1016/j.intimp.2018.02.004]

115   **Romach E**, Uni Z, Friedman M, Aizenberg I, Berkovich Z, Reifen R. A new mode of probiotic therapy: Specific targeting. *J Funct Foods* 2015; **16**: 386-392 [DOI: 10.1016/j.jff.2015.04.029]

116   **Cencic A**, Chingwaru W. The role of functional foods, nutraceuticals, and food supplements in intestinal health. *Nutrients* 2010; **2**: 611-625 [PMID: 22254045 DOI: 10.3390/nu2060611]

Advances in immuno-oncology biomarkers for gastroesophageal cancer: Programmed death ligand 1, microsatellite instability, and beyond

Emily M Lin, Jun Gong, Samuel J Klempner, Joseph Chao

Emily M Lin, Department of Internal Medicine, Harbor-UCLA Medical Center, Torrance, CA 90509, United States

Jun Gong, Joseph Chao, Department of Medical Oncology and Developmental Therapeutics, City of Hope Comprehensive Cancer Center, Duarte, CA 91010, United States

Samuel J Klempner, The Angeles Clinic and Research Institute, Los Angeles, CA 90404, United States

Samuel J Klempner, Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048, United States

ORCID number: Emily M Lin (0000-0003-2166-3201); Jun Gong (0000-0001-8713-1406); Samuel J Klempner (0000-0002-4062-0808); Joseph Chao (0000-0002-1809-504X).

Author contributions: Lin EM, Gong J and Chao J contributed to conception and design, provision of study materials, and collection and assembly of data; all authors contributed to data analysis and interpretation, manuscript writing, and final approval of manuscript.

Manuscript source: Invited manuscript

Correspondence to: Joseph Chao, MD, Assistant Professor, Department of Medical Oncology and Therapeutics Research, City of Hope Comprehensive Cancer Center, 1500 E. Duarte Rd., Duarte, CA 91010, United States. jchao@coh.org
Telephone: +1-626-4719200
Fax: +1-626-3018233

Abstract

Blockade of the programmed death ligand 1 (PD-L1) and programmed cell death 1 (PD-1) receptor axis represents an effective form of cancer immunotherapy. Preclinical evidence initially suggested that gastric and gastroesophageal junction (GEJ) cancers are potentially immunotherapy-sensitive tumors. Early phase clinical trials have demonstrated promising antitumor activity with PD-1/PD-L1 blockade in advanced or metastatic gastric/GEJ cancer. Microsatellite instability (MSI) and PD-L1 expression have been shown to predict higher response to PD-1 inhibitors as highlighted by the recent approvals of pembrolizumab in treatment-refractory solid tumors with MSI status and the third-line or greater treatment of PD-L1 positive advanced gastric/GEJ cancers. However, predictive and prognostic biomarkers remain an ongoing need. In this review, we detail the preclinical evidence and early tissue biomarker analyses illustrating potential predictive biomarkers to PD-1/PD-L1 blockade in gastric/GEJ cancer. We also review the clinical development of PD-1/PD-L1 inhibitors in gastric/GEJ cancer and

highlight several areas in need of future investigation in order to optimize the efficacy of PD-1/PD-L1 blockade in gastric/GEJ cancer.

Key words: Immunotherapy; Programmed cell death 1; Programmed death ligand 1; Microsatellite instablility; Gastric cancer

Core tip: Programmed death ligand 1 (PD-L1) and microsatellite instability have recently entered into clinical practice as recommended biomarker testing for the use of immune checkpoint inhibitors in gastroesophageal cancer. However, PD-L1 still does not carry the highest sensitivity and specificity with variability in testing reported. Incorporation of PD-L1 expression from the tumor microenvironment with counting of immune cells appears to be the most effective strategy to date. Future efforts focusing on composite biomarkers in ongoing research from combinatorial immuno-oncology strategies are necessary to drive the field forward.

INTRODUCTION

T-cell activation and tolerance are partly regulated by the B7 family of proteins[1]. B7-H1, a transmembrane protein in the B7 family also known as programmed death ligand 1 (PD-L1), has been shown to negatively regulate T-cell-mediated immune responses when bound by the programmed cell death 1 (PD-1) receptor[1-3]. Deficiency of PD-1 leads to impaired tolerance, as demonstrated by the development of pathologies resembling lupus and graft-versus-host disease in mice with PD-1 gene disruption, and in transgenic mice with the PD-1 null mutation, respectively[4].

Many tumors upregulate PD-L1 expression, which can be enhanced by interferon-gamma signaling[3,5]. PD-L1 expression leads to apoptosis of tumor-reactive T cells and tumor growth[5]. Tumorigenesis and tumor dissemination are also increased, but these effects were reversed when anti-PD-L1 antibody was administered[6]. Therefore, activation of the PD-1/PD-L1 axis was a putative mechanism for tumors to evade host tumor antigen-specific T-cell immunity, leading to the concept of PD-1/PD-L1 blockade as a potential form of cancer immunotherapy[3,5,6]. Initial phase I studies of

humanized monoclonal IgG4 antibodies against PD-1 and PD-L1 in patients with advanced solid tumors were soon conducted, demonstrating both tumor shrinkage and extended disease stabilization[7]. This paved the way for the development of the first Food and Drug Administration (FDA)-approved PD-1 inhibitors, pembrolizumab and nivolumab, which achieved durable objective responses in early trials[7-9].

Cancers of the stomach and esophagus are among the 8 major global cancers that account for > 60% of total cases and deaths worldwide[10]. In 2012, an estimated 1.4 million cases of gastroesophageal cancer were reported, resulting in 1.1 million deaths globally. Approximately 2/3 of patients with gastroesophageal cancer develop metastatic disease during the course of their disease and despite sequencing of available active systemic agents, prognosis remains poor in advanced disease with a median overall survival (OS) of 8-10 mo[11]. Despite the rapidly growing number of FDA approvals for PD-1/PD-L1 inhibitors in cancer therapy, the first approval of a PD-1 inhibitor specifically for advanced gastroesophageal cancer occurred on September 22, 2017 with the approval of pembrolizumab[12]. Likewise, the PD-1 inhibitor nivolumab received concurrent regulatory approval in Japan for unresectable advanced or recurrent gastric cancer[13]. Although clinical activity is established, biomarkers to optimize patient selection remain an area of significant need in gastroesophageal cancers.

In this review, we highlight the clinical development of PD-1 inhibitors leading up to the recent approvals of pembrolizumab and nivolumab in advanced gastroesophageal cancer. In particular, we discuss preclinical rationale, early biomarker studies, and results currently available from major phase I-III trials investigating PD-1/PD-L1 inhibitors in advanced or metastatic gastroesophageal cancer.

INITIAL STUDIES INTO THE IMMUNOGENICITY OF GASTROESOPHAGEAL CANCER

Landmark analyses by The Cancer Genome Atlas (TCGA) proposed classifications based on comprehensive genomic profiling for 4 subtypes of gastric cancer: Epstein-Barr virus (EBV)-infection, microsatellite instability (MSI), genomic stability, and chromosomal instability[14]. Similarly, classifications were proposed for 3 molecular subtypes of esophageal squamous cell carcinoma (SCC)[15]. Of particular interest were findings suggestive that a relevant proportion of gastric cancer cases may be inherently receptive to immune checkpoint blockade, given that EBV-positive subtypes representing 9% of cases were characterized by genomic amplification of chromosomal region 9p24.1, the locus of genes encoding PD-L1 and PD-L2, and 21.7% of cases demonstrated MSI[14]. In-silico analyses

from RNA-sequencing data also identified gastric cancers as one of the tumor types associated with immune cytolytic activity[16].

A meta-analysis recently demonstrated that tumor and tumor-infiltrating immune cell PD-L1 expression by immunohistochemistry (IHC) is predictive of response to PD-1/PD-L1 inhibitors [odds ratio (OR) 2.26, 95% confidence interval (CI): 1.85-2.75, $P < 0.001$] among advanced solid tumors studied across 41 trials[17]. However, growing evidence suggests that PD-L1 expression alone as the sole predictor of response to PD-1/PD-L1 blockade may not be sufficient, given the lack of response still observed in some PD-L1-expressing tumors, and response in PD-L1 negative patients[18]. Furthermore, there is increasing focus on immune properties of the tumor microenvironment (TME) including density of CD8+ tumor-infiltrating lymphocytes (TILs), expression of various immune checkpoints, and other immune cell phenotypes that may serve as predictive biomarkers for PD-1/PD-L1 blockade[18-21]. Analyses of PD-L1 expression and the TME in gastroesophageal cancers, however, have been limited and only recently have investigations begun to report findings on these topics.

Many studies have focused on quantifying PD-L1 expression and its clinical significance among gastroesophageal cancers. Among histological types of esophageal cancers, SCCs were observed to have higher PD-L1 expression[22]. In another study, presence of TILs and PD-L2 in esophageal cancers were inversely correlated, in contrast to PD-L1 expression, which had no significant correlation with TILs[23]. PD-L1 positivity, however, was associated with significantly poorer prognosis - especially in more advanced stages - and found to be an independent prognostic factor upon multivariate analysis[23].

PD-L1 is not expressed by normal gastric tissue[24], and either not expressed or weakly expressed by gastric adenomas[24,25]. However, 30%-65% of invasive gastric cancers express PD-L1[25-30], and expression was found to correlate to depth of invasion, lymph node metastasis, distant metastasis, and tumor size[24,26,27,31,32]. EBV-positive gastric cancers had higher rates of PD-L1 expression in tumor and immune cells more often than EBV-negative gastric cancers[29,33-36]. In particular, Derks *et al*[29] found that among EBV-positive gastric cancers from the TCGA dataset, PD-L1 was expressed in immune cells in 94% of the cases, whereas only 50% of the cases had tumor cell expression of PD-L1. Among EBV-negative gastric cancers, only those with MSI were found to express PD-L1 within tumor cells. However, EBV-negative cancers without MSI had inflammatory cell expression of PD-L1 in 35% of the cases, and these inflammatory cells were present only at the invasive margin as opposed to deeply infiltrating the tumor. Interestingly, findings of tumor-infiltrating PD-L1+ inflammatory cells occurred only in cancers with

EBV positivity or MSI[29], and among gastric cancers in another study, these were noted to have upregulated immune escape pathway genes[34]. Mismatch repair (MMR) deficiency has also been associated with PD-L1 expression in other series[30,37].

The relationship between other immune checkpoint molecules and PD-1/PD-L1 among gastric cancers has also been an increasing focus of interest. Expression of FOXP3, a transcription factor involved in regulatory T cell (Treg) function and development, correlated to PD-1 expression among patients with stages II and III gastric cancers[38]. Another study found significant correlation between FOXP3+ Tregs and PD-L1 expression, and significantly higher expression of both was found in patients with more advanced clinicopathological stage and lymph node metastasis; patients with higher levels of FOXP3+ Tregs and PD-L1 expression had poorer prognosis[39]. Blood levels of both PD-1 and the molecule T-cell immunoglobulin-3 (Tim-3), which downregulates T helper 1 and cytotoxic cells, were elevated in gastric cancer patients[40]. In addition, PD-1+ and Tim-3+ CD8 T cells produced less IFN-gamma compared to PD-1 negative- and Tim-3-negative cells, suggestive of T-cell dysfunction[40-42]. In a gastric cancer surgical series, post-operative circulating CD4+ and CD8+ T-cells were found to upregulate PD-1 and lymphocyte activation gene 3 (LAG-3), another co-inhibitor of T-cell activation[43]. Gastric cancer tumor cells have also been reported to more commonly express cytotoxic T-lymphocyte antigen 4 (CTLA-4), a major immune checkpoint molecule with known therapeutic strategies, than PD-L1 (86.7% *vs* 44.9%, respectively)[44]. However, gastric cancer TILs expressed more PD-L1 and PD-1 than CTLA-4[44].

Investigation of PD-1/PD-L1 expression among TILs and the TME has also grown. Gastric cancer expression of PD-L1 was associated with TILs that were positive for CD3, CD8, or FOXP3[45]. PD-L1+ gastric cancers tended to have stromal immune cells expressing PD-1 and PD-L1, and those with PD-L1+ immune cells had increased depth of invasion, although PD-L1+ tumor cells had greater prognostic impact than did PD-L1+ immune cells[36]. Although both PD-L1 expression and increased CD3+ TIL density in the TME of gastric cancers were significantly associated with improved 5-year disease-free survival (DFS) and OS, there was no significant correlation between PD-L1 expression and CD3+ TIL density, leading to the hypothesis that tumor production of immunosuppressive proteins may be a mechanism intrinsic to the tumor[46]. Among gastric and GEJ adenocarcinomas, the majority (44%) expressed PD-L1 in the immune stroma, whereas a minority (12%) expressed PD-L1 on tumor cell membranes[47]. Increased density of CD8+ T cells was associated with PD-L1 expression, as well as with worse progression-free and overall survival, suggestive of adaptive immune resistance[47]. In a study of gastric signet-

Table 1 Phase I clinical trials of programmed cell death 1 inhibitors involving advanced gastroesophageal cancer

n	Primary tumor	Doses	Primary endpoint	Results	Ref.
277[1]	NSCLC, melanoma, cutaneous, mucosal, ocular, RCC, clear cell, non-clear cell, other (CRC, gastric, esophageal, HNSCC, sarcoma, ovarian, breast, pancreatic, uterine, pancreaticoduodenal)	Atezolizumab at escalating doses up to 20 mg/kg every 3 wk	Safety, tolerability, DLT, and RP2D	13% grade 3-4 TRAEs: 5 fatigue; 3 each of increased ALT, increased AST, hypoxia; 2 each of asthenia, dyspnea, myalgia, anemia, hyperglycemia, hyponatremia, cardiac tamponade, hypophosphatemia, tumor lysis syndrome; 1 each of nausea, headache, influenza-like illness, pain, vomiting ORR 18% overall; 21% of NSCLC, 26% of melanoma, 13% of RCC, and 13% of other malignancies (CRC, gastric, HNSCC)	[49]
151	Gastric or GEJ	Avelumab (MSB0010718C) 10 mg/kg every 2 wk until progression, toxicity, or withdrawal	Safety, efficacy	9.9% TRAEs grade ≥ 3: fatigue, asthenia, increased GGT, thrombocytopenia, anemia; 1 treat-ment-related death 14 patients with unconfirmed response: 9.7% patients on 2nd line therapy (all PRs), 9.0% patients on 1st-line maintenance (2 CRs, 6 PRs); disease control rate 29% for 2nd line, 57.3% for 1st line maintenance	JAVELIN [50]
39	PD-L1+ Gastric (previously treated)	Pembrolizumab 10 mg/kg every 2 wk for 2 yr or PD	Safety, tolerability, ORR	13% grade 3-4 TRAEs: 2 grade 3 fatigue, 1 each of grade 3 pemphigoid, hypothyroidism, neuropathy, and 1 grade 4 pneumonitis ORR 22% (95%CI: 10-39)	KEYNOTE 012 [51]
23	PD-L1+ SCC or adenocarcinoma of esophagus or GEJ	Pembrolizumab 10 mg/kg every 2 wk up to 2 yr or until PD, intolerable toxicity, or investigator decision	Safety, ORR	17.4% grade 3-4 TRAEs: 2 with decreased lymphocytes, other 2 patients AE was not specified ORR 30.4% (95%CI: 13.2%-52.9%)	KEYNOTE 028 [53]

[1]Note that 175 patients were "efficacy-evaluable". PD: Progressive disease; SD: Stable disease; ORR: Overall response rate; TRAE: Treatment-related adverse effects; DCR: Disease control rate; DLT: Dose limiting toxicities; RP2D: Recommended phase 2 dose.

ring cell carcinoma, a histologic subtype historically associated with poor prognosis, the presence of CD3+ TILs was associated with increased expression of PD-1 and PD-L1, presence of MSI, and an improved OS[48].

PD-L1 BIOMARKER ANALYSES FROM PHASE I TRIALS

Multiple early phase clinical trials of immune checkpoint inhibitors incorporated cohorts of gastroesophageal cancers to establish early signals of anti-tumor activity and exploratory biomarker analyses (Table 1). The anti-PD-L1 antibody atezolizumab was studied in multiple malignancies including gastroesophageal cancer and found to have grade 3-4 TRAEs in 13% of patients as well as an overall response rate[10] of 18%[49]. PD-L1

expression by IHC was determined using an anti-human PD-L1 rabbit monoclonal antibody (clone SP142; Ventana, Tucson, AZ, United States), with the authors scoring both tumor cells and immune cells. Response was significantly associated with the presence of PD-L1 positivity in tumor-infiltrating immune cells (*P* = 0.007), but not tumor cells (*P* = 0.079), with better response among patients with greater IHC scores. Among 141 gastric cancer cases included in the trial, 18% demonstrated PD-L1 expression in immune cells, compared with 5% demonstrating PD-L1 expression in tumor cells. Likewise, the JAVELIN phase Ib trial examined the anti-PD-L1 inhibitor avelumab as first-line maintenance or second-line therapy in a gastric and GEJ cancer cohort[50]. The study demonstrated a disease control rate (DCR) of 57.3% and 29.0% in first- and second-line therapy, respectively. Patients receiving

avelumab exhibited increased ORR if harboring PD-L1 positivity of at least 1% tumor cell staining by an IHC assay (Dako, clone 73-10). However, responses were also observed even in cases with PD-L1 expression < 1% albeit at a lower proportion. TRAEs of grade 3 or higher occurred in 9.9%, with one treatment-related death due to hepatic failure.

KEYNOTE 012 was an open-label trial across 13 centers that investigated pembrolizumab for previously treated gastric cancer[51]. Presence of tumor PD-L1 expression was a requirement for enrollment, with 40% of the patients screened demonstrating PD-L1-positive tumors. PD-L1 expression was detected using the 22C3 antibody with a prototype assay using QualTek or Dako platforms. Tumor positivity was based on a cutoff of at least 1% of scorable tumor cells or immune cells exhibiting membrane staining, or the presence of PD-L1-positive mononuclear inflammatory cells existing in the interface between tumor and stromal cells. ORR was 22% (95%CI: 10-39) comprised exclusively of partial responses[52] as no complete responses (CRs) were observed. Median progression-free survival (PFS) was 1.9 mo (95%CI: 1.8-3.5) and median OS was 11.4 mo (95%CI: 5.7-not reached). 44% of patients with a mononuclear inflammatory cell density of 3 had PR, whereas a 0-2 density score corresponded to a PR rate of 15%. Focusing on tumor cell staining, 24% of patients with a tumor score of 0 had a response, compared to 17% of those with a score of at least 1 (Table 1). As such, there did not appear to be an absolute lower cutoff that could reliably exclude the possibility of response. KEYNOTE 028 investigated pembrolizumab in patients with advanced solid malignancy also requiring tumor expression of PD-L1 as detected by IHC in tumor or stroma, including those with SCC or adenocarcinoma of the esophagus or GEJ[53]. Among esophageal cancers screened, 45% met the criteria for PD-L1 expression. For the patients treated, 13.0% attained stable disease[31], and ORR was reported at 30.4%, with a PFS of 30.4% and 21.7% at 6 and 12 mo, respectively (Table 1). Median duration of response (DOR) was 40.0 wk.

PD-L1 BIOMARKER ANALYSES FROM PHASE II TRIALS

Combination immunotherapy with nivolumab, an anti-PD-1 antibody, and ipilimumab, an anti-CTLA-4 antibody, was tested for advanced gastric, esophageal, and GEJ adenocarcinoma in the phase I/II CheckMate 032 trial (Table 2). Patients received nivolumab alone, nivolumab 1 mg/kg with ipilimumab 3 mg/kg (N1+I3), or nivolumab 3mg/kg with ipilimumab 1mg/kg (N3+I1)[54]. Grade 3-4 TRAEs including diarrhea and elevated transaminases were reported more often in the N1+I3 group, which also achieved greater ORR regardless of PD-L1 status. PD-L1 IHC expression was determined by examining solely tumor cell expression with staining by the 28-8 antibody. 39 of 127 assessable cases (31%) exhibited PD-L1 expression ≥ 1%. While ORRs were greater in PD-L1 ≥ 1% tumors (19% nivolumab alone, 40% N1+I3, 23% N3+I1), responses were still demonstrable in PD-L1 < 1% tumors (12% nivolumab alone, 22% N1+I3, 0% N3+I1). Survival outcomes reported to date have not demonstrated clear differences in median OS between the 3 arms, but as a phase II trial the study was not powered to address this difference. PD-L1 ≥ 1% tumors appeared to demonstrate more favorable rates of 12-month OS in the N1+I3 (50%) *vs* nivolumab alone (34%) and N3+I1 (23%) groups. However, with the small numbers of PD-L1 ≥ 1% tumors in each arm (16 nivolumab alone, 10 N1+I3, 13 N3+I1), it remains difficult to conclude if PD-L1 IHC assessment of tumor cells alone is robust enough to enrich for gastroesophageal cancer patients who will benefit from immune checkpoint inhibitors.

KEYNOTE 059 investigated pembrolizumab among 3 cohorts of patients with gastric and GEJ cancer: (Cohort 1) pembrolizumab after at least 2 prior regimens, (Cohort 2) pembrolizumab with cisplatin and 5-fluorouracil or capecitabine as first-line therapy, and (Cohort 3) pembrolizumab as first-line therapy among patients with at least 1% PD-L1 expression as scored by the 22C3 IHC pharmDx assay[55]. PD-L1 positivity was defined in this study as a combined positive score (CPS) ≥ 1 where the number of PD-L1 positive tumor and immune cells (lymphocytes and macrophages) were divided by the total number of tumor cells evaluated and multiplied by 100. Cohort 1 comprised the largest cohort of 259 patients, and found that those with a CPS ≥ 1 (*n* = 148, 57%) had an ORR 15.5%, comprised of a CR rate of 2.0% and PR rate of 13.5%. Those considered to be PD-L1 negative, i.e. a CPS < 1 (*n* = 109), had an ORR of 6.4%, and still exhibited CRs at a rate of 2.8% and PR rate of 3.7%[56]. As such, lack of detectable PD-L1 tumor and/or immune cell expression did not completely exclude the possibility of deriving a response, including CR in 2.8%, though likelihood of response appeared higher if PD-L1 expression was detected. PD-L1 CPS-positive patients had a median DOR of 16.3 mo, compared to 6.9 mo in PD-L1 CPS-negative patients. 51.7% had 2 prior therapies, 29.0% had 3 prior therapies, and 19.3% had 4 or more prior therapies. Patients receiving pembrolizumab as third-line therapy had greater response, with a 16.4% ORR and 3.0% CR rate, compared to those receiving fourth-line therapy[56]. PD-L1-negative patients had an ORR of 5.5%, 1.8% CRs, and 3.7% PRs. Overall 16.6% had grade 3-5 TRAEs, leading to therapy discontinuation and death in 2 patients each.

Cohort 2, which received combined frontline chemotherapy with pembrolizumab, demonstrated a median PFS of 6.6 mo and median OS of 13.8 mo

Table 2 Phase II and III clinical trials of programmed cell death 1 inhibitors in advanced gastroesophageal cancer

n (phase)	Experimental arm	Control or reference arm	Primary endpoint	Results	Ref.
160 (I/II)	N1 + I3: Nivolumab 1 mg/kg every 2 wk and ipilimumab 3 mg/kg every 3 wk N3 + I1: Nivolumab 3 mg/kg and ipilimumab 1 mg/kg every 3 wk Gastric, esophageal, or GEJ cancer	N3: Nivolumab 3 mg/kg every 2 wk	ORR	N3: ORR 12%, PD-L1 ≥ 1% ORR 19%, PD-L1< 1% ORR 12% N1+I3: ORR 24%, PD-L1≥ 1% ORR 40%, PD-L1< 1% ORR 22% N3+I1: ORR 8%, PD-L1 ≥ 1% ORR 23%, PD-L1 < 1% ORR 0%	CheckMate 032 [54]
259 (II)	Cohort 1 (after ≥ 2 lines of therapy): Pembrolizumab 200 mg every 3 wk up to 2 yr, PD, decision to withdraw, or unacceptable toxicity in gastric cancer	N/A	ORR, safety, tolerability	Overall ORR 11.2% (95%CI: 7.6-15.7), CR 1.9% (95%CI: 0.6-4.4), PR 9.3% (95%CI: 6.0-13.5), SD 17% (95%CI: 12.6-22.1), PD 55.6% (95% CI 49.3-61.7) PD-L1+ ORR 15.5% (95% CI 10.1-22.4), PD-L1- ORR 5.5% (95% CI 2.0-11.6)	KEYNOTE 059 [56]
25 (II)	Cohort 2 (1st line): pembrolizumab 200 mg every 3 wk for up to 2 yr, cisplatin (80 mg/m² day 1), and 5-FU (800 mg/m² D1-5 Q3W) or capecitabine (1000 mg/ m² bid)	N/A	Safety, ORR	Cohort 2: ORR 60% (39-79) overall, 73% (45-92) PD-L1+, 38% (9-76) PD-L1-. Median PFS 7 mo	KEYNOTE 059 [55]
31 (II)	Cohort 3 (PD-L1+, 1st line): pembrolizumab 200 mg every 3 wk for up to 2 yr Gastric or GEJ cancer	N/A	Safety, ORR	Cohort 3: ORR 26% (12-45). Median PFS 3 mo	KEYNOTE 059 [55]
41 (II)	Pembrolizumab 10 mg/kg every 2 wk Cohort A: Mismatch repair (MMR)-deficient colorectal cancers (CRC) Cohort C: MMR-deficient non-CRC	Cohort B: MMR-proficient CRC	ORR, PFS	MMR-deficient CRC: ORR 40%, PFS 78%; median PFS and OS not reached MMR-proficient CRC: ORR 0%, PFS 11%; median PFS 2.2 mo, OS 5.0 mo MMR-deficient non-CRC: ORR 71%, PFS 67%	Keynote-016 [58]
86 (II)	Pembrolizumab 10 mg/kg every 2 wk for MMR-deficient cancers (12 tumor types)	N/A	ORR, PFS	Objective radiographic response 53%, CR 21%; median PFS and OS not reached	[59]
493 (III)	Nivolumab 3 mg/kg every 2 wk until unacceptable toxicity or PD in gastric/GEJ cancers	Placebo	OS	Nivolumab: median OS 5.32 mo, 6-mo OS 46.4%, 12-mo OS 26.6%, ORR 11.2%, median PFS 1.61 mo Placebo: median OS 4.14 mo, 6-mo OS 34.7%, 12-mo OS 10.9%, ORR 0%, median PFS 1.45 mo	ATTRACTION-02 [57]

PD: Progressive disease; ORR: Overall response rate; CI: Confidence interval; OS: Overall survival.

among this smaller cohort of 25 patients. PD-L1 CPS-positive patients had an ORR of 69%, compared to 38% for PD-L1 CPS-negative patients. Thus, the latter appeared in line with historical trials of doublet platinum and fluoropyrimidine chemotherapy in advanced gastric cancer. The presence of PD-L1 CPS positivity would suggest that the addition of pembrolizumab can help achieve deeper responses with chemotherapy. However, this should be cautiously interpreted with the limited number of patients analyzed. Cohort 3, which was single-agent pembrolizumab in first-line therapy but

selecting for tumors that had PD-L1 CPS ≥ 1, led to a promising ORR of 26%, median PFS of 3.3 mo, and a median OS of 20.7 mo. Grade 3-5 TRAEs occurred in 18% (cohort 1), 76% (cohort 2), and 23% (cohort 3), with cohort 2 not unexpectedly demonstrating more toxicities associated with use of cytotoxic chemotherapy. Findings from cohort 1 of KEYNOTE 059 ultimately led to the accelerated FDA approval of pembrolizumab in third-line and beyond treatment of PD-L1 positive by the CPS criteria advanced or metastatic gastric or GEJ adenocarcinoma in September of 2017[12].

PD-L1 BIOMARKER ANALYSIS FROM PHASE III TRIALS

Nivolumab was investigated in a phase III trial of 493 patients with gastric and GEJ cancer who had advanced or recurrent disease after 2 or more lines of chemotherapy[57]. Patients receiving nivolumab had significantly improved median OS of 5.2 mo, PFS 1.61 mo, and ORR 11.2%, compared to 4.14 mo, 1.45 mo, and 0%, respectively in patients receiving placebo (all $P < 0.0001$). 11.5% of the nivolumab group and 5.5% of the placebo group suffered TRAEs of grade 3 or higher. An exploratory analysis was conducted of PD-L1 biomarker expression using the 28-8 antibody for IHC and defining PD-L1 positivity as staining in ≥ 1% of tumor cells only. Tumor testing was able to be retrospectively conducted on 192 patient samples, with 26 patients (14%) harboring tumors with PD-L1 positivity in ≥ 1% of tumor cells. Median OS appeared improved with nivolumab vs. placebo regardless of PD-L1 positive (5.22 mo *vs* 3.83 mo) or PD-L1 negative (6.05 mo *vs* 4.19 mo) tumor status. As such, definitive conclusions on tumor PD-L1 status influencing the likelihood of benefit from nivolumab therapy could not be drawn with the limited numbers of PD-L1 positive tumors observed. Subsequently, nivolumab garnered regulatory approval in Japan for third-line and beyond therapy for advanced gastric cancer irrespective of PD-L1 biomarker testing.

MSI AS A BIOMARKER IN GASTRIC CANCER

The seminal phase II trial by Le *et al*[58] demonstrated metastatic colorectal cancers (CRCs) which demonstrated deficient DNA mismatch repair (dMMR) and harbored MSI had a higher propensity to respond to pembrolizumab in comparison to CRCs with proficient MMR and were microsatellite stable (MSS). It is now well recognized that dMMR tumors harbor a high mutational burden translating into the production of tumor neoantigens which evade immune response through the upregulation of immune checkpoints[59]. In addition to dMMR and pMMR CRCs, they included a third cohort of dMMR tumors regardless of histology. Both dMMR CRC and non-CRC cohorts demonstrated encouraging ORRs of 40% and 67%, respectively. Le *et al*[60] subsequently expanded the study further to 86 patients inclusive of 12 tumor types with dMMR, which continued to demonstrate a high ORR of 53% and CR rate of 21%, with median PFS and OS not reached at the time of reporting. Gastroesophageal cancers among 5 patients were included as one of the 12 tumor histologies. Three of the 5 patients exhibited CRs, while 2 of 5 had progressive disease to comprise an ORR of 60%. The high and durable ORRs of pembrolizumab in MSI-high (MSI-H) non-CRC tumors also led to efforts to retrospectively identify patients with MSI-H

gastroesophageal cancers from the KEYNOTE-012 and KEYNOTE-028 trials. Pooling of these datasets with the study by Le *et al*[60] in addition to the MSI-H cohort of the ongoing KEYNOTE-158 umbrella trial (NCT02628067), subsequently led to the identification of 9 gastric/GEJ adenocarcinoma patients with reportable responses[61]. Among the 9 cases, 5 (56%) demonstrated an objective response, with median DOR ranging from 5.8 mo to 22.1 mo and ongoing at last analysis. These studies eventually culminated in the unprecedented FDA approval of pembrolizumab in treatment-refractory MSI-H solid tumors, regardless of tissue histology[62].

Cohort 1 of the KEYNOTE-059 trial also conducted an analysis for microsatellite instability, and among 174 assessable cases, 7 were MSI-H (4%). Of these 7 MSI-H gastric/GEJ cancers, the ORR was 57.1% (14.3% CR, 42.9% PR), and a disease control rate (DCR) of 71.4% was also achieved[56]. In contrast, the non-MSI-H subset had an ORR of 9.0% (2.4% CRs, 6.6% PRs) and DCR of 22.2%. Given that MSI-H status and PD-L1 positivity tend to occur together, it is likely that all MSI-H patients in this cohort were PD-L1 positive. In assuming that 4/7 of these MSI-H patients would have responded to pembrolizumab (ORR of 57.1%), subtracting these cases from the PD-L1+ cohort only marginally affects the ORR to pembrolizumab in this cohort (13.5% from the original 15.5%). MSI biomarker analysis was also conducted retrospectively in the gastroesophageal cohorts of the Checkmate 032 trial[63]. Among 72 assessable cases, 11 (15%) were considered MSI-H tumors. Seven of the cases belonged in the nivolumab alone cohort, and 2 each in the N1+I3 and N3+I1 cohorts, thus no definitive comparison could be made of responses in MSI-H cases among the 3 treatment arms. Regardless, ORR was 2/7 (29%) with nivolumab alone, 1/2 (50%) with N1+I3, and 1/2 (50%) with N3+I1. Similar to the data from KEYNOTE-059, the DCR with nivolumab alone among MSI-H gastroesophageal tumors was 71%. Among non-MSI-H tumors, the ORR was 11% with nivolumab alone, 19% with N1+I3, and 5% with N3+I1.

In conclusion, MSI is present in a small, but clinically relevant proportion of gastroesophageal cancers. Responses to PD-1 inhibitors appear to be more favorable in this subset from the small numbers of patients reported in the literature to date. However, lack of MSI does not exclude the potential for response, and it is apparent that primary resistance in MSI-H tumors still exists by the reported cases with no evidence of response to immune checkpoint inhibitors.

MOVING BEYOND PD-L1 AND MSI BIOMARKERS

Other potential predictive biomarkers are gaining interest, and advances in determining prognosis with the assistance of immune markers are ongoing. Dai *et al*[64]

studied the prognostic value of multiple methods of immune marker detection in stage I-IV gastric cancers. PD-L1 expression and TIL infiltration were evaluated by IHC; PD-L1 expression was associated with higher TIL density, and TIL density correlated to lower rates of disease progression and improved survival. Increased mRNA levels of multiple immune markers including PD-L1, CTLA-4, FOXP3, and LAG-3, as detected by real-time quantitative polymerase chain reaction were found in patients with better OS. In-situ hybridization demonstrated a correlation between EBV positivity and PD-L1 expression as well as higher TIL infiltration.

Tumor mutational burden (TMB) has also been of interest as a predictive biomarker for immunotherapy strategies in multiple other tumors[49,65,66]. MSI-H tumors with defective dMMR certainly exemplify high TMB with analyses by Llosa *et al*[59] reporting on average 1782 mutations per tumor by whole-exome sequencing in cases of MMR deficiency, compared to 73 in cases with MMR proficiency ($P = 0.007$). Such findings translated into clinical benefit with an association between greater mutation rates and prolonged PFS with pembrolizumab ($P = 0.02$). Clinically available targeted next-generation sequencing panels have also reliably captured TMB in gastrointestinal malignancies[67]. Klempner *et al*[67] reported from a large case series of tumors sequenced using the FoundationOne platform (Cambridge, MA, United Kingdom) that high TMB, defined as > 20 mutations/Mb, existed in 3% of 2065 esophageal cases and 5% of 1485 stomach cancers. Ongoing investigation is required to determine whether there is an ideal TMB cutoff that predicts high likelihood of response to immune checkpoint inhibition in gastroesophageal cancer. An early report from Ku *et al*[68] utilizing the Memorial Sloan Kettering IMPACT panel suggested that having ≥ 14 mutations/Mb corresponded to greater benefit from immune checkpoint inhibition (2-year OS rate 15% *vs* 60%, $P = 0.094$). However, the proportion of patients comprising this high TMB subset was small (6/55 patients), with 4 of the 6 being comprised of dMMR tumors[68]. The frequency of other genomic alterations which lead to hypermutated phenotypes such as *POLE* mutations have also been characterized in gastroesophageal cancers, but appear to comprise an even lower proportion (< 1%) than dMMR[67]. A follow-up report of gastroesophageal cancers sequenced by the IMPACT panel appeared to suggest that a cutoff of > 9.7 mutations/Mb, representing the top quartile of 40 patients treated with immune checkpoint inhibitors, correlated to greater benefit (median OS 16.8 mo *vs* 6.62 mo, $P = 0.058$)[69]. Interestingly, 2 patients with durable responses lasting > 12 mo had low TMB (1.9 and 3.3 mutations/Mb), with one of the cases being EBV+. As such, the detection of low TMB does not appear to entirely exclude response to immune checkpoint inhibitors. A recent phase I trial in on the anti-PD-1 antibody SHR-1210 in ESCC supported somatic nonsynonymous mutational load (by WES) as a biomarker of predictive benefit with higher TMB associated with benefit ($P = 0.048$)[70].

With greater understanding of the dynamics of immune signaling necessary for antitumor responses, immune gene expression profiling has also entered into the forefront of biomarker analyses. High throughput assays such as the NanoString platform (Seattle, WA, United States) were applied to the early KEYNOTE-012 gastric dataset in attempts to enrich for tumor biomarkers beyond PD-L1 expression to be predictive of pembrolizumab response[51]. Muro *et al*[71] reported that high scores from a 6-gene interferon γ signature (*STAT1, HLA-DRA, IFNG, IDO1, CXCL9, CXCL10*) correlated to response, but conclusive results were limited by small patient numbers. Likewise, the same 6-gene signature was examined in the KEYNOTE-028 esophageal dataset, and a correlation to pembrolizumab response was also observed with higher scores. Lastly, Fuchs *et al*[56] reported from the larger dataset of cohort 1 from KEYNOTE-059 that a higher score from an 18-gene T-cell inflamed signature (*CCL5, CD27, CD274 (PD-L1), CD276 (B7-H3), CD8A, CMKLR1, CXCL9, CXCR6, HLA-DQA1, HLA-DRB1, HLA-E, IDO1, LAG3, NKG7, PDCD1LG2 (PD-L2), PSMB10, STAT1, TIGIT*) was significantly associated with improved response to pembrolizumab ($P = 0.014$). Thus, immune gene expression profiling in conjunction with PD-L1 IHC scoring may provide greater specificity to predicting response to anti-PD-1 therapy.

Characterization of gastric cancers has become more complex, with the availability of a variety of techniques such as multiplex IHC, in-situ hybridization, and gene expression profiling. These have expanded the field of immune therapy and facilitated the study of relationships between PD-1/PD-L1 and other factors including EBV positivity, MSI, TIL density, and the presence of other immune markers. The rapidly growing recognition of the stool microbiome influencing immunotherapy responses adds additional complexity from environmental factors influencing biomarker analyses[52,72,73]. Further investigation into these variables in advanced gastric/GEJ cancer is duly warranted to assist further development of immunotherapeutic efforts. With currently available therapies in gastric/GEJ cancer, as in other cancers, a composite score incorporating other immune checkpoints, TILs, MSI status, tumor mutational burden, and the immune profile of the TME may represent a more robust predictive biomarker for checkpoint inhibitors rather than PD-L1 expression alone. Furthermore, to optimize the antitumor efficacy of PD-1/PD-L1 inhibitors in gastric cancer, trials have been conducted and are ongoing to examine the potential of PD-1/PD-L1 inhibitors in combination with other therapies. While combination strategies are likely necessary, it may come at a cost of additive toxicity, and as such robust predictive biomarkers will truly allow for personalization of immunotherapeutic approaches.

As observed in all of the trials discussed, the majority of patients do not derive significant benefit and demonstrate primary resistance to currently studied immune checkpoint inhibitors. Identification of patients least likely to respond is equally important to optimal

response biomarker studies. Whether or not high disease burden, low mutational burden, or the IPRES (innate anti-PD-1 resistance) transcriptional signature seen in melanoma can serve as surrogates for resistance (or have a mechanistic role) in gastric and esophageal cancers remains unknown[74-76]. Studies in the non-metastatic setting, many of which are ongoing, may aid in identifying some resistance markers and changes between locoregional and advanced disease[77].

CONCLUSION

Cancer immunotherapeutic approaches with PD-1/PD-L1 inhibitors continue to gain considerable momentum as more disease indications are added. Preclinical and early biomarker studies provided ample evidence that gastric/GEJ cancer is an immune-sensitive tumor. Immune parameters including MSI status, TILs, PD-L1 expression, and the immune profile of the TME are among some, but not all, of the potential predictive biomarkers for checkpoint inhibitors in advanced gastric/GEJ cancer. Early phase clinical trials provided promising signals of antitumor activity of PD-1/PD-L1 blockade to advance into larger studies. Recently, pembrolizumab received FDA approval for the third-line treatment of PD-L1 CPS expressing advanced gastric/GEJ adenocarcinoma. With the CPS criterion, this represents the first biomarker testing incorporating both assaying of tumor cells and the associated immune cells within the TME. Pembrolizumab has also been approved in a tissue-agnostic indication for treatment-refractory solid tumors that are MSI-H. In the small proportion of gastroesophageal cancers harboring this biomarker, encouraging and clinically relevant responses have been reported in the few cases reported to date. Nivolumab likewise has received Japanese approval for third-line treatment in advanced gastric/GEJ adenocarcinoma, though a predictive biomarker has not been linked to this indication. We anticipate composite biomarkers will improve patient selection and potentially individualize treatment, though broader clinical implementation may be slow. The identification of more robust predictive biomarkers and development of combination therapies incorporating immune checkpoint inhibitors represent necessary and ongoing areas of investigation to optimize this class of agents in gastric/GEJ cancer.

REFERENCES

1   **Dong H**, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med* 1999; **5**: 1365-1369 [PMID: 10581077 DOI: 10.1038/70932]

2   **Freeman GJ**, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, Horton HF, Fouser L, Carter L, Ling V, Bowman MR, Carreno BM, Collins M, Wood CR, Honjo T. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 2000; **192**: 1027-1034 [PMID: 11015443]

3   **Greenwald RJ**, Freeman GJ, Sharpe AH. The B7 family revisited. *Annu Rev Immunol* 2005; **23**: 515-548 [PMID: 15771580 DOI: 10.1146/annurev.immunol.23.021704.115611]

4   **Nishimura H**, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 1999; **11**: 141-151 [PMID: 10485649]

5   **Dong H**, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, Lennon VA, Celis E, Chen L. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 2002; **8**: 793-800 [PMID: 12091876 DOI: 10.1038/nm730]

6   **Iwai Y**, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci USA* 2002; **99**: 12293-12297 [PMID: 12218188 DOI: 10.1073/pnas.192461099]

7   **Brahmer JR**, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, Pitot HC, Hamid O, Bhatia S, Martins R, Eaton K, Chen S, Salay TM, Alaparthy S, Grosso JF, Korman AJ, Parker SM, Agrawal S, Goldberg SM, Pardoll DM, Gupta A, Wigginton JM. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med* 2012; **366**: 2455-2465 [PMID: 22658128 DOI: 10.1056/NEJMoa1200694]

8   **Patnaik A**, Kang SP, Rasco D, Papadopoulos KP, Elassaiss-Schaap J, Beeram M, Drengler R, Chen C, Smith L, Espino G, Gergich K, Delgado L, Daud A, Lindia JA, Li XN, Pierce RH, Yearley JH, Wu D, Laterza O, Lehnert M, Iannone R, Tolcher AW. Phase I Study of Pembrolizumab (MK-3475; Anti-PD-1 Monoclonal Antibody) in Patients with Advanced Solid Tumors. *Clin Cancer Res* 2015; **21**: 4286-4293 [PMID: 25977344 DOI: 10.1158/1078-0432.ccr-14-2607]

9   **Topalian SL**, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 2012; **366**: 2443-2454 [PMID: 22658127 DOI: 10.1056/NEJMoa1200690]

10   **Torre LA**, Siegel RL, Ward EM, Jemal A. Global Cancer Incidence and Mortality Rates and Trends--An Update. *Cancer Epidemiol Biomarkers Prev* 2016; **25**: 16-27 [PMID: 26667886 DOI: 10.1158/1055-9965.epi-15-0578]

11   **Shah MA**. Update on metastatic gastric and esophageal cancers. *J Clin Oncol* 2015; **33**: 1760-1769 [PMID: 25918288 DOI: 10.1200/jco.2014.60.1799]

12   **U.S. Food Drug Administration**. FDA grants accelerated approval to pembrolizumab for advanced gastric cancer. September 22, 2017 ed, 2017

13   **Squibb BM**. Japan Ministry of Health, Labor and Welfare Approves Opdivo (nivolumab) for the Treatment of Patients with Unresectable Advanced or Recurrent Gastric Cancer Which Has Progressed After Chemotherapy. 2017

14   **Cancer Genome Atlas Research Network**. Comprehensive molecular characterization of gastric adenocarcinoma. *Nature* 2014; **513**: 202-209 [PMID: 25079317 DOI: 10.1038/nature13480]

15   **Cancer Genome Atlas Research Network**; Analysis Working Group: Asan University; BC Cancer Agency; Brigham and Women's Hospital; Broad Institute; Brown University; Case Western Reserve University; Dana-Farber Cancer Institute; Duke University; Greater Poland Cancer Centre; Harvard Medical School; Institute for Systems Biology; KU Leuven; Mayo Clinic; Memorial Sloan Kettering Cancer Center; National Cancer Institute; Nationwide Children's Hospital; Stanford University; University of Alabama; University of Michigan; University of North Carolina; University of Pittsburgh; University of Rochester; University of Southern California; University of Texas MD Anderson Cancer Center; University of Washington; Van Andel Research Institute; Vanderbilt University; Washington University; Genome Sequencing Center:

Broad Institute; Washington University in St. Louis; Genome Characterization Centers: BC Cancer Agency; Broad Institute; Harvard Medical School; Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University; University of North Carolina; University of Southern California Epigenome Center; University of Texas MD Anderson Cancer Center; Van Andel Research Institute; Genome Data Analysis Centers: Broad Institute; Brown University:; Harvard Medical School; Institute for Systems Biology; Memorial Sloan Kettering Cancer Center; University of California Santa Cruz; University of Texas MD Anderson Cancer Center; Biospecimen Core Resource: International Genomics Consortium; Research Institute at Nationwide Children's Hospital; Tissue Source Sites: Analytic Biologic Services; Asan Medical Center; Asterand Bioscience; Barretos Cancer Hospital; BioreclamationIVT; Botkin Municipal Clinic; Chonnam National University Medical School; Christiana Care Health System; Cureline; Duke University; Emory University; Erasmus University; Indiana University School of Medicine; Institute of Oncology of Moldova; International Genomics Consortium; Invidumed; Israelitisches Krankenhaus Hamburg; Keimyung University School of Medicine; Memorial Sloan Kettering Cancer Center; National Cancer Center Goyang; Ontario Tumour Bank; Peter MacCallum Cancer Centre; Pusan National University Medical School; Ribeirão Preto Medical School; St. Joseph's Hospital &Medical Center; St. Petersburg Academic University; Tayside Tissue Bank; University of Dundee; University of Kansas Medical Center; University of Michigan; University of North Carolina at Chapel Hill; University of Pittsburgh School of Medicine; University of Texas MD Anderson Cancer Center; Disease Working Group: Duke University; Memorial Sloan Kettering Cancer Center; National Cancer Institute; University of Texas MD Anderson Cancer Center; Yonsei University College of Medicine; Data Coordination Center: CSRA Inc; Project Team: National Institutes of Health. Integrated genomic characterization of oesophageal carcinoma. *Nature* 2017; **541**: 169-175 [PMID: 28052061 DOI: 10.1038/nature20805]

16   **Rooney MS**, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell* 2015; **160**: 48-61 [PMID: 25594174 DOI: 10.1016/j.cell.2014.12.033]

17   **Khunger M**, Hernandez AV, Pasupuleti V, Rakshit S, Pennell NA, Stevenson J, Mukhopadhyay S, Schalper K, Velcheti V. Programmed cell death 1 (PD-1) ligand (PD-L1) expression in solid tumors as a predictive biomarker of benefit from PD-1/PD-L1 axis inhibitors: A systematic review and meta-analysis. *JCO Precision Oncol* 2017: 1-15 [DOI: 10.1200/PO.1216.00030]

18   **Gibney GT**, Weiner LM, Atkins MB. Predictive biomarkers for checkpoint inhibitor-based immunotherapy. *Lancet Oncol* 2016; **17**: e542-e551 [PMID: 27924752 DOI: 10.1016/s1470-2045(16)30406-5]

19   **Chen YP**, Zhang Y, Lv JW, Li YQ, Wang YQ, He QM, Yang XJ, Sun Y, Mao YP, Yun JP, Liu N, Ma J. Genomic Analysis of Tumor Microenvironment Immune Types across 14 Solid Cancer Types: Immunotherapeutic Implications. *Theranostics* 2017; **7**: 3585-3594 [PMID: 28912897 DOI: 10.7150/thno.21471]

20   **Iglesia MD**, Parker JS, Hoadley KA, Serody JS, Perou CM, Vincent BG. Genomic Analysis of Immune Cell Infiltrates Across 11 Tumor Types. *J Natl Cancer Inst* 2016; **108** [PMID: 27335052 DOI: 10.1093/jnci/djw144]

21   **Taube JM**, Klein A, Brahmer JR, Xu H, Pan X, Kim JH, Chen L, Pardoll DM, Topalian SL, Anders RA. Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. *Clin Cancer Res* 2014; **20**: 5064-5074 [PMID: 24714771 DOI: 10.1158/1078-0432.ccr-13-3271]

22   **Grosso J**, Inzunza D, Wu Q, Simon J, Singh P, Zhang X, Phillips T, Simmons P, Cogswell J. Programmed death-ligand 1 (PD-L1) expression in various tumor types [abstract]. *J Immunother Cancer* 2013; **1**: P53 [DOI: 10.1186/2051-1426-1-S1-P53]

23   **Ohigashi Y**, Sho M, Yamada Y, Tsurui Y, Hamada K, Ikeda N, Mizuno T, Yoriki R, Kashizuka H, Yane K, Tsushima F, Otsuki N, Yagita H, Azuma M, Nakajima Y. Clinical significance of programmed death-1 ligand-1 and programmed death-1 ligand-2 expression in human esophageal cancer. *Clin Cancer Res* 2005; **11**: 2947-2953 [PMID: 15837746 DOI: 10.1158/1078-0432.ccr-04-1469]

24   **Sun J**, Xu K, Wu C, Wang Y, Hu Y, Zhu Y, Chen Y, Shi Q, Yu G, Zhang X. PD-L1 expression analysis in gastric carcinoma tissue and blocking of tumor-associated PD-L1 signaling by two functional monoclonal antibodies. *Tissue Antigens* 2007; **69**: 19-27 [PMID: 17212704 DOI: 10.1111/j.1399-0039.2006.00701.x]

25   **Wu C**, Zhu Y, Jiang J, Zhao J, Zhang XG, Xu N. Immunohistochemical localization of programmed death-1 ligand-1 (PD-L1) in gastric carcinoma and its clinical significance. *Acta Histochem* 2006; **108**: 19-24 [PMID: 16530813 DOI: 10.1016/j.acthis.2006.01.003]

26   **Geng Y**, Wang H, Lu C, Li Q, Xu B, Jiang J, Wu C. Expression of costimulatory molecules B7-H1, B7-H4 and Foxp3+ Tregs in gastric cancer and its clinical significance. *Int J Clin Oncol* 2015; **20**: 273-281 [PMID: 24804867 DOI: 10.1007/s10147-014-0701-7]

27   **Zhang L**, Qiu M, Jin Y, Ji J, Li B, Wang X, Yan S, Xu R, Yang D. Programmed cell death ligand 1 (PD-L1) expression on gastric cancer and its relationship with clinicopathologic factors. *Int J Clin Exp Pathol* 2015; **8**: 11084-11091 [PMID: 26617827]

28   **Böger C**, Behrens HM, Mathiak M, Krüger S, Kalthoff H, Röcken C. PD-L1 is an independent prognostic predictor in gastric cancer of Western patients. *Oncotarget* 2016; **7**: 24269-24283 [PMID: 27009855 DOI: 10.18632/oncotarget.8169]

29   **Derks S**, Liao X, Chiaravalli AM, Xu X, Camargo MC, Solcia E, Sessa F, Fleitas T, Freeman GJ, Rodig SJ, Rabkin CS, Bass AJ. Abundant PD-L1 expression in Epstein-Barr Virus-infected gastric cancers. *Oncotarget* 2016; **7**: 32925-32932 [PMID: 27147580 DOI: 10.18632/oncotarget.9076]

30   **Kimura K**, Nagasaka T, Mori Y, Kawai T, Fuji T, Taniguchi F, Yasui K, Toshima T, Umeda Y, Tazawa H, Goel A, Fujiwara T. Abstract 2317: PD-1 and PD-L1 expression patterns and DNA mismatch repair status for precision management of patients with gastric cancer. *Cancer Res* 2016; **76**: Abstr 2317 [DOI: 10.1158/1538-7445.AM2016-2317]

31   **Liu SM**, Meng Q, Zhang QX, Wang SD, Liu ZJ, Zhang XF. [Expression and significance of B7-H1 and its receptor PD-1 in human gastric carcinoma]. *Zhonghua Zhong Liu Za Zhi* 2008; **30**: 192-195 [PMID: 18756934]

32   **Qing Y**, Li Q, Ren T, Xia W, Peng Y, Liu GL, Luo H, Yang YX, Dai XY, Zhou SF, Wang D. Upregulation of PD-L1 and APE1 is associated with tumorigenesis and poor prognosis of gastric cancer. *Drug Des Devel Ther* 2015; **9**: 901-909 [PMID: 25733810 DOI: 10.2147/dddt.s75152]

33   **Ma C**, Patel K, Singhi AD, Ren B, Zhu B, Shaikh F, Sun W. Programmed Death-Ligand 1 Expression Is Common in Gastric Cancer Associated With Epstein-Barr Virus or Microsatellite Instability. *Am J Surg Pathol* 2016; **40**: 1496-1506 [PMID: 27465786 DOI: 10.1097/pas.0000000000000698]

34   **Wang K**, Leung SY. Abstract PR06: Genomic characterization of immune escape pathways in gastric cancer. *Cancer Immunol Res* 2015; **3**: Abstr PR06 [DOI: 10.1158/2326-6074.TUMIMM14-PR06]

35   **Dong M**, Wang HY, Zhao XX, Chen JN, Zhang YW, Huang Y, Xue L, Li HG, Du H, Wu XY, Shao CK. Expression and prognostic roles of PIK3CA, JAK2, PD-L1, and PD-L2 in Epstein-Barr virus-associated gastric carcinoma. *Hum Pathol* 2016; **53**: 25-34 [PMID: 26980034 DOI: 10.1016/j.humpath.2016.02.007]

36   **Saito R**, Abe H, Kunita A, Yamashita H, Seto Y, Fukayama M. Overexpression and gene amplification of PD-L1 in cancer cells and PD-L1+ immune cells in Epstein-Barr virus-associated gastric cancer: the prognostic implications. *Mod Pathol* 2017; **30**: 427-439 [PMID: 27934877 DOI: 10.1038/modpathol.2016.202]

37   **Kim ST**, Klempner SJ, Park SH, Park JO, Park YS, Lim HY, Kang WK, Kim KM, Lee J. Correlating programmed death ligand 1 (PD-L1) expression, mismatch repair deficiency, and outcomes across tumor types: implications for immunotherapy. *Oncotarget* 2017; **8**: 77415-77423 [PMID: 29100397 DOI: 10.18632/oncotarget.20492]

38   **Eto S**, Yoshikawa K, Nishi M, Higashijima J, Tokunaga T, Nakao T, Kashihara H, Takasu C, Iwata T, Shimada M. Programmed cell death protein 1 expression is an independent prognostic factor in gastric cancer after curative resection. *Gastric Cancer* 2016; **19**: 466-471 [PMID: 26210691 DOI: 10.1007/s10120-015-0519-7]

39   **Hou J**, Yu Z, Xiang R, Li C, Wang L, Chen S, Li Q, Chen M, Wang L. Correlation between infiltration of FOXP3+ regulatory T cells and expression of B7-H1 in the tumor tissues of gastric cancer. *Exp Mol Pathol* 2014; **96**: 284-291 [PMID: 24657498 DOI: 10.1016/j.yexmp.2014.03.005]

40   **Takano S**, Saito H, Ikeguchi M. An increased number of PD-1+ and Tim-3+ CD8+ T cells is involved in immune evasion in gastric cancer. *Surg Today* 2016; **46**: 1341-1347 [PMID: 26801344 DOI: 10.1007/s00595-016-1305-9]

41   **Saito H**, Kuroda H, Matsunaga T, Osaki T, Ikeguchi M. Increased PD-1 expression on CD4+ and CD8+ T cells is involved in immune evasion in gastric cancer. *J Surg Oncol* 2013; **107**: 517-522 [PMID: 23129549 DOI: 10.1002/jso.23281]

42   **Lu X**, Yang L, Yao D, Wu X, Li J, Liu X, Deng L, Huang C, Wang Y, Li D, Liu J. Tumor antigen-specific CD8+ T cells are negatively regulated by PD-1 and Tim-3 in human gastric cancer. *Cell Immunol* 2017; **313**: 43-51 [PMID: 28110884 DOI: 10.1016/j.cellimm.2017.01.001]

43   **Takaya S**, Saito H, Ikeguchi M. Upregulation of Immune Checkpoint Molecules, PD-1 and LAG-3, on CD4+ and CD8+ T Cells after Gastric Cancer Surgery. *Yonago Acta Med* 2015; **58**: 39-44 [PMID: 26190896]

44   **Schlößer HA**, Drebber U, Kloth M, Thelen M, Rothschild SI, Haase S, Garcia-Marquez M, Wennhold K, Berlth F, Urbanski A, Alakus H, Schauss A, Shimabukuro-Vornhagen A, Theurich S, Warnecke-Ebertz U, Stippel DL, Zippelius A, Büttner R, Hallek M, Hölscher AH, Zander T, Mönig SP, von Bergwelt-Baildon M. Immune checkpoints programmed death 1 ligand 1 and cytotoxic T lymphocyte associated molecule 4 in gastric adenocarcinoma. *Oncoimmunology* 2015; **5**: e1100789 [PMID: 27467911 DOI: 10.1080/2162402x.2015.1100789]

45   **Kawazoe A**, Kuwata T, Kuboki Y, Shitara K, Nagatsuma AK, Aizawa M, Yoshino T, Doi T, Ohtsu A, Ochiai A. Clinicopathological features of programmed death ligand 1 expression with tumor-infiltrating lymphocyte, mismatch repair, and Epstein-Barr virus status in a large cohort of gastric cancer patients. *Gastric Cancer* 2017; **20**: 407-415 [PMID: 27629881 DOI: 10.1007/s10120-016-0631-3]

46   **Kim JW**, Nam KH, Ahn SH, Park DJ, Kim HH, Kim SH, Chang H, Lee JO, Kim YJ, Lee HS, Kim JH, Bang SM, Lee JS, Lee KW. Prognostic implications of immunosuppressive protein expression in tumors as well as immune cell infiltration within the tumor microenvironment in gastric cancer. *Gastric Cancer* 2016; **19**: 42-52 [PMID: 25424150 DOI: 10.1007/s10120-014-0440-5]

47   **Thompson ED**, Zahurak M, Murphy A, Cornish T, Cuka N, Abdelfatah E, Yang S, Duncan M, Ahuja N, Taube JM, Anders RA, Kelly RJ. Patterns of PD-L1 expression and CD8 T cell infiltration in gastric adenocarcinomas and associated immune stroma. *Gut* 2017; **66**: 794-801 [PMID: 26801886 DOI: 10.1136/gutjnl-2015-310839]

48   **Jin S**, Xu B, Yu L, Fu Y, Wu H, Fan X, Wei J, Liu B. The PD-1, PD-L1 expression and CD3+ T cell infiltration in relation to outcome in advanced gastric signet-ring cell carcinoma, representing a potential biomarker for immunotherapy. *Oncotarget* 2017; **8**: 38850-38862 [PMID: 28418918 DOI: 10.18632/oncotarget.16407]

49   **Herbst RS**, Soria JC, Kowanetz M, Fine GD, Hamid O, Gordon MS, Sosman JA, McDermott DF, Powderly JD, Gettinger SN, Kohrt HE, Horn L, Lawrence DP, Rost S, Leabman M, Xiao Y, Mokatrin A, Koeppen H, Hegde PS, Mellman I, Chen DS, Hodi FS. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature* 2014; **515**: 563-567 [PMID: 25428504 DOI: 10.1038/nature14011]

50   **Chung HC**, Arkenau HT, Wyrwicz L, Oh DY, Lee KW, Infante JR, Lee SS, Lee J, Keilholz U, Mita AC, Plummer ER, Kemeny M, Melichar B, Smith DM, Chin KM, von Heydebreck A, Cuillerot JM, Kang YK, Safran H. Avelumab (MSB0010718C; anti-PD-L1)

in patients with advanced gastric or gastroesophageal junction cancer from JAVELIN solid tumor phase Ib trial: Analysis of safety and clinical activity. *J Clin Oncol* 2016; **34**: 4009 [DOI: 10.1200/JCO.2016.34.15_suppl.4009]

51   **Muro K**, Chung HC, Shankaran V, Geva R, Catenacci D, Gupta S, Eder JP, Golan T, Le DT, Burtness B, McRee AJ, Lin CC, Pathiraja K, Lunceford J, Emancipator K, Juco J, Koshiji M, Bang YJ. Pembrolizumab for patients with PD-L1-positive advanced gastric cancer (KEYNOTE-012): a multicentre, open-label, phase 1b trial. *Lancet Oncol* 2016; **17**: 717-726 [PMID: 27157491 DOI: 10.1016/s1470-2045(16)00175-3]

52   **Gopalakrishnan V**, Spencer CN, Nezi L, Reuben A, Andrews MC, Karpinets TV, Prieto PA, Vicente D, Hoffman K, Wei SC, Cogdill AP, Zhao L, Hudgens CW, Hutchinson DS, Manzo T, Petaccia de Macedo M, Cotechini T, Kumar T, Chen WS, Reddy SM, Szczepaniak Sloane R, Galloway-Pena J, Jiang H, Chen PL, Shpall EJ, Rezvani K, Alousi AM, Chemaly RF, Shelburne S, Vence LM, Okhuysen PC, Jensen VB, Swennes AG, McAllister F, Marcelo Riquelme Sanchez E, Zhang Y, Le Chatelier E, Zitvogel L, Pons N, Austin-Breneman JL, Haydu LE, Burton EM, Gardner JM, Sirmans E, Hu J, Lazar AJ, Tsujikawa T, Diab A, Tawbi H, Glitza IC, Hwu WJ, Patel SP, Woodman SE, Amaria RN, Davies MA, Gershenwald JE, Hwu P, Lee JE, Zhang J, Coussens LM, Cooper ZA, Futreal PA, Daniel CR, Ajami NJ, Petrosino JF, Tetzlaff MT, Sharma P, Allison JP, Jenq RR, Wargo JA. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. *Science* 2018; **359**: 97-103 [PMID: 29097493 DOI: 10.1126/science.aan4236]

53   **Doi T**, Piha-Paul SA, Jalal SI, Mai-Dang H, Saraf S, Koshiji M, Csiki I, Bennouna J. Updated results for the advanced esophageal carcinoma cohort of the phase 1b KEYNOTE-028 study of pembrolizumab. *J Clin Oncol* 2016; **34**: Abstr 4046 [DOI: 10.1200/jco.2016.34.4_suppl.7]

54   **Janjigian YY**, Ott PA, Calvo E, Kim JW, Ascierto PA, Sharma P, Peltola KJ, Jaeger D, Evans TRJ, De Braud FG, Chau I, Tschaika M, Harbison CT, Cai W, Bendell JC, Le DT. Nivolumab ± ipilimumab in pts with advanced (adv)/metastatic chemotherapy-refractory (CTx-R) gastric (G), esophageal (E), or gastroesophageal junction (GEJ) cancer: CheckMate 032 study. *J Clin Oncol* 2017; **35**: Abstr 4014 [DOI: 10.1200/JCO.2017.35.15_suppl.4014]

55   **Wainberg ZA**, Jalal S, Muro K, Yoon HH, Garrido M, Golan T, Doi T, Catenacci DV, Geva R, Ku G, Bleeker J, Bang YJ, Hara H, Chung HC, Savage M, Wang J, Koshiji M, Dalal R, Fuchs CS. KEYNOTE-059 Update: Efficacy and safety of pembrolizumab alone or in combination with chemotherapy in patients with advanced gastric or gastroesophageal (G/GEJ) cancer. *Ann Oncol* 2017; **28**: LBA28_PR [DOI: 10.1093/annonc/mdx440.020]

56   **Fuchs CS**, Doi T, Jang RW, Muro K, Satoh T, Machado M, Sun W, Jalal SI, Shah MA, Metges JP, Garrido M, Golan T, Mandala M, Wainberg ZA, Catenacci DVT, Ohtsu A, Shitara K, Geva R, Bleeker J, Ko AH, Ku G, Philip P, Enzinger PC, Bang YJ, Levitan D, Wang J, Rosales M, Dalal RP, Yoon HH. Safety and efficacy of pembrolizumab monotherapy in patients with previously treated advanced gastric and gastroesophageal junction cancer: Phase 2 clinical KEYNOTE-059 trial. *JAMA Oncol* 2018; **4**: e180013 [PMID: 29543932 DOI: 10.1001/jamaoncol.2018.0013]

57   **Kang YK**, Satoh T, Ryu MH, Chao Y, Kato K, Chung HC, Chen JS, Muro K, Kang WK, Yoshikawa T, Oh SC, Tamura T, Lee KW, Boku N, Chen LT. Nivolumab (ONO-4538/BMS-936558) as salvage treatment after second or later-line chemotherapy for advanced gastric or gastro-esophageal junction cancer (AGC): A double-blinded, randomized, phase III trial. *J Clin Oncol* 2017; **35**: Abstract 02 [DOI: 10.1200/JCO.2017.35.4_suppl.2]

58   **Le DT**, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, Skora AD, Luber BS, Azad NS, Laheru D, Biedrzycki B, Donehower RC, Zaheer A, Fisher GA, Crocenzi TS, Lee JJ, Duffy SM, Goldberg RM, de la Chapelle A, Koshiji M, Bhaijee F, Huebner T, Hruban RH, Wood LD, Cuka N, Pardoll DM, Papadopoulos N, Kinzler KW, Zhou S, Cornish TC, Taube JM, Anders RA, Eshleman JR, Vogelstein B, Diaz LA Jr. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med* 2015; **372**: 2509-2520 [PMID: 26028255 DOI: 10.1056/NEJMoa1500596]

59 **Llosa NJ**, Cruise M, Tam A, Wicks EC, Hechenbleikner EM, Taube JM, Blosser RL, Fan H, Wang H, Luber BS, Zhang M, Papadopoulos N, Kinzler KW, Vogelstein B, Sears CL, Anders RA, Pardoll DM, Housseau F. The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. *Cancer Discov* 2015; **5**: 43-51 [PMID: 25358689 DOI: 10.1158/2159-8290.CD-14-0863]

60 **Le DT**, Durham JN, Smith KN, Wang H, Bartlett BR, Aulakh LK, Lu S, Kemberling H, Wilt C, Luber BS, Wong F, Azad NS, Rucki AA, Laheru D, Donehower R, Zaheer A, Fisher GA, Crocenzi TS, Lee JJ, Greten TF, Duffy AG, Ciombor KK, Eyring AD, Lam BH, Joe A, Kang SP, Holdhoff M, Danilova L, Cope L, Meyer C, Zhou S, Goldberg RM, Armstrong DK, Bever KM, Fader AN, Taube J, Housseau F, Spetzler D, Xiao N, Pardoll DM, Papadopoulos N, Kinzler KW, Eshleman JR, Vogelstein B, Anders RA, Diaz LA Jr. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science* 2017; **357**: 409-413 [PMID: 28596308 DOI: 10.1126/science.aan6733]

61 **Zhang W**, Pang Q, Yan C, Wang Q, Yang J, Yu S, Liu X, Yuan Z, Wang P, Xiao Z. Induction of PD-L1 expression by epidermal growth factor receptor-mediated signaling in esophageal squamous cell carcinoma. *Onco Targets Ther* 2017; **10**: 763-771 [PMID: 28243112 DOI: 10.2147/ott.s118982]

62 **U.S. Food Drug Administration.** FDA approves first cancer treatment for any solid tumor with a specific genetic feature. May 24, 2017 ed, 2017

63 **Ott PA**, Le DT, Kim JW, Ascierto PA, Sharma P, Bono P, Peltola K, Jäger D, Evans TRJ, de Braud F, Chau I, Bendell JC, Tschaika M, Harbison CT, Zhao H, Calvo E, Janjigian Y. Nivolumab (NIVO) in patients (pts) with advanced (adv) chemotherapy-refractory (CT-Rx) esophagogastric (EG) cancer according to microsatellite instability (MSI) status: checkmate 032. *Ann Oncol* 2017; **28**: 674P [DOI: 10.1093/annonc/mdx369.058]

64 **Dai C**, Geng R, Wang C, Wong A, Qing M, Hu J, Sun Y, Lo AW, Li J. Concordance of immune checkpoints within tumor immune contexture and their prognostic significance in gastric cancer. *Mol Oncol* 2016; **10**: 1551-1558 [PMID: 27720576 DOI: 10.1016/j.molonc.2016.09.004]

65 **Rizvi NA**, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, Lee W, Yuan J, Wong P, Ho TS, Miller ML, Rekhtman N, Moreira AL, Ibrahim F, Bruggeman C, Gasmi B, Zappasodi R, Maeda Y, Sander C, Garon EB, Merghoub T, Wolchok JD, Schumacher TN, Chan TA. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science* 2015; **348**: 124-128 [PMID: 25765070 DOI: 10.1126/science.aaa1348]

66 **Gong J**, Chehrazi-Raffle A, Reddi S, Salgia R. Development of PD-1 and PD-L1 inhibitors as a form of cancer immunotherapy: a comprehensive review of registration trials and future considerations. *J Immunother Cancer* 2018; **6**: 8 [PMID: 29357948 DOI: 10.1186/s40425-018-0316-z]

67 **Klempner SJ**, Frampton GM, Chao J, Bailey M, Stephens P, Ross JS, Miller VA, Ali SM, Schrock AB. Topography of Tumor Mutational Burden (TMB) and Immune-related Genomic Alterations (GA) Across Gastrointestinal Malignancies (GIm): A Study of 22,570 Cases. *Ann Oncol* 2017; **28**: 1169P [DOI: 10.1093/annonc/mdx376.034]

68 **Ku GY**, Sanchez-Vega F, Chatila W, Margolis M, Fein C, Ilson DH, Hechtman JF, Tuvy Y, Bouvier N, Kundra R, Shia J, Heins ZJ, Middha S, Zehir A, Ladanyi M, Berger MF, Tang LH, Solit DB, Schultz N, Janjigian YY. Correlation of benefit from immune checkpoint inhibitors with next gen sequencing (NGS) profiles in esophagogastric cancer (EGC) patients. *J Clin Oncol* 2017; **35**: 4025-4025 [DOI: 10.1200/JCO.2017.35.15_suppl.4025]

69 **Janjigian YY**, Sanchez-Vega F, Jonsson P, Chatila WK, Hechtman JF, Ku GY, Riches JC, Tuvy Y, Kundra R, Bouvier N, Vakiani E, Gao J, Heins ZJ, Gross BE, Kelsen DP, Zhang L, Strong VE, Schattner M, Gerdes H, Coit DG, Bains M, Stadler ZK, Rusch VW, Jones DR, Molena D, Shia J, Robson ME, Capanu M, Middha S, Zehir A, Hyman DM, Scaltriti M, Ladanyi M, Rosen N, Ilson DH, Berger MF, Tang L, Taylor BS, Solit DB, Schultz N. Genetic Predictors of Response to Systemic Therapy in Esophagogastric Cancer. *Cancer Discov* 2018; **8**: 49-58 [PMID: 29122777 DOI: 10.1158/2159-8290.CD-17-0787]

70 **Huang J**, Xu B, Mo H, Zhang W, Chen X, Wu D, Qu D, Wang X, Lan B, Yang B, Wang P, Zhang H, Yang Q, Jiao Y. Safety, Activity, and Biomarkers of SHR-1210, an Anti-PD-1 Antibody, for Patients with Advanced Esophageal Carcinoma. *Clin Cancer Res* 2018; **24**: 1296-1304 [PMID: 29358502 DOI: 10.1158/1078-0432.CCR-17-2439]

71 **Doi T**, Piha-Paul SA, Jalal SI, Saraf S, Lunceford J, Koshiji M, Bennouna J. Safety and Antitumor Activity of the Anti-Programmed Death-1 Antibody Pembrolizumab in Patients With Advanced Esophageal Carcinoma. *J Clin Oncol* 2018; **36**: 61-67 [PMID: 29116900 DOI: 10.1200/JCO.2017.74.9846]

72 **Matson V**, Fessler J, Bao R, Chongsuwat T, Zha Y, Alegre ML, Luke JJ, Gajewski TF. The commensal microbiome is associated with anti-PD-1 efficacy in metastatic melanoma patients. *Science* 2018; **359**: 104-108 [PMID: 29302014 DOI: 10.1126/science.aao3290]

73 **Routy B**, Le Chatelier E, Derosa L, Duong CPM, Alou MT, Daillère R, Fluckiger A, Messaoudene M, Rauber C, Roberti MP, Fidelle M, Flament C, Poirier-Colame V, Opolon P, Klein C, Iribarren K, Mondragón L, Jacquelot N, Qu B, Ferrere G, Clémenson C, Mezquita L, Masip JR, Naltet C, Brosseau S, Kaderbhai C, Richard C, Rizvi H, Levenez F, Galleron N, Quinquis B, Pons N, Ryffel B, Minard-Colin V, Gonin P, Soria JC, Deutsch E, Loriot Y, Ghiringhelli F, Zalcman G, Goldwasser F, Escudier B, Hellmann MD, Eggermont A, Raoult D, Albiges L, Kroemer G, Zitvogel L. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. *Science* 2018; **359**: 91-97 [PMID: 29097494 DOI: 10.1126/science.aan3706]

74 **Nishino M**, Giobbie-Hurder A, Manos MP, Bailey N, Buchbinder EI, Ott PA, Ramaiya NH, Hodi FS. Immune-Related Tumor Response Dynamics in Melanoma Patients Treated with Pembrolizumab: Identifying Markers for Clinical Outcome and Treatment Decisions. *Clin Cancer Res* 2017; **23**: 4671-4679 [PMID: 28592629 DOI: 10.1158/1078-0432.CCR-17-0114]

75 **Snyder A**, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, Walsh LA, Postow MA, Wong P, Ho TS, Hollmann TJ, Bruggeman C, Kannan K, Li Y, Elipenahli C, Liu C, Harbison CT, Wang L, Ribas A, Wolchok JD, Chan TA. Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N Engl J Med* 2014; **371**: 2189-2199 [PMID: 25409260 DOI: 10.1056/NEJMoa1406498]

76 **Hugo W**, Zaretsky JM, Sun L, Song C, Moreno BH, Hu-Lieskovan S, Berent-Maoz B, Pang J, Chmielowski B, Cherry G, Seja E, Lomeli S, Kong X, Kelley MC, Sosman JA, Johnson DB, Ribas A, Lo RS. Genomic and Transcriptomic Features of Response to Anti-PD-1 Therapy in Metastatic Melanoma. *Cell* 2016; **165**: 35-44 [PMID: 26997480 DOI: 10.1016/j.cell.2016.02.065]

77 **Murugaesu N**, Wilson GA, Birkbak NJ, Watkins T, McGranahan N, Kumar S, Abbassi-Ghadi N, Salm M, Mitter R, Horswell S, Rowan A, Phillimore B, Biggs J, Begum S, Matthews N, Hochhauser D, Hanna GB, Swanton C. Tracking the genomic evolution of esophageal adenocarcinoma through neoadjuvant chemotherapy. *Cancer Discov* 2015; **5**: 821-831 [PMID: 26003801 DOI: 10.1158/2159-8290.CD-15-0412]

MINIREVIEWS

Minimally invasive donor hepatectomy, are we ready for prime time?

Kin Pan Au, Kenneth Siu Ho Chok

Kin Pan Au, Department of Surgery, Queen Mary Hospital, Hong Kong, China

Kenneth Siu Ho Chok, Department of Surgery and State Key Laboratory for Liver Research, The University of Hong Kong, Hong Kong, China

ORCID number: Kin Pan Au (0000-0002-7138-9805); Kenneth Siu Ho Chok (0000-0001-7921-3807).

Abstract

Minimally invasive surgery potentially reduces operative morbidities. However, pure laparoscopic approaches to donor hepatectomy have been limited by technical complexity and concerns over donor safety. Reduced-wound donor hepatectomy, either in the form of a laparoscopic-assisted technique or by utilizing a mini-laparotomy wound, *i.e.*, hybrid approach, has been developed to bridge the transition to pure laparoscopic donor hepatectomy, offering some advantages of minimally invasive surgery. To date, pure laparoscopic donor left lateral sectionectomy has been validated for its safety and advantages and has become the standard in experienced centres. Pure laparoscopic approaches to major left and right liver donation have been reported for their technical feasibility in expert hands. Robotic-assisted donor hepatectomy also appears to be a valuable alternative to pure laparoscopic donor hepatectomy, providing additional ergonomic advantages to the surgeon. Existing reports derive from centres with tremendous experience in both laparoscopic hepatectomy and donor hepatectomy. The complexity of these procedures means an arduous transition from technical feasibility to reproducibility. Donor safety is paramount in living donor liver transplantation. Careful donor selection and adopting standardized techniques allow experienced transplant surgeons to safely accumulate experience and acquire proficiency. An international prospective registry will advance the understanding for the role and safety of pure laparoscopic donor hepatectomy.

Key words: Laparoscopic donor hepatectomy; Living donor liver transplantation; Minimally invasive surgery

Core tip: Reduced-wound donor hepatectomy has been developed to bridge the transition to pure laparoscopic

donor hepatectomy, offering some advantages of minimally invasive surgery. To date, pure laparoscopic donor left lateral sectionectomy has been validated for its safety and advantages, while pure laparoscopic approaches to major left and right liver donation have been reported for their feasibility in expert hands. Careful donor selection and adopting standardized techniques allow experienced transplant surgeons to accumulate experience in this complex procedure. An international prospective registry will advance the understanding for the role and safety of pure laparoscopic donor hepatectomy.

INTRODUCTION

Liver transplantation is the most effective treatment for end-stage liver disease. Shortage of cadaveric grafts has encouraged the rapid development of living donor liver transplantation (LDLT). However, donor morbidity remains the primary concern and the major bottleneck for LDLT. Donor operation poses a 25%-35% morbidity[1,2] to a healthy individual. and half of morbidities are related to abdominal wall trauma, including hernia, intestinal obstruction and chronic wound pain[2]. Permanent large incision brings physical and mental stress to young women.

On the other hand, the minimally invasive approach to liver resection has gained wide acceptance for oncological indications[3]. Laparoscopic hepatectomy has been carried out for liver tumours with minimal mortality and morbidity[4]. Various reviews and meta-analyses have validated the benefits of this technique, which include reduced blood loss, less postoperative pain and hastened recovery[5-11]. Considering the advantages of laparoscopic hepatectomy, it appears legitimate to transfer these benefits to liver donors. To such an end, minimally invasive donor hepatectomy was introduced to reduce the morbidity of open hepatectomy[12]. However, the development of minimally invasive donor hepatectomy has advanced at a slow and arduous pace. The first pure laparoscopic right liver donation[13] was reported only 15 years after the first laparoscopic right hepatectomy[14]. Concerns still exist regarding the safety and outcomes for minimally invasive donor hepatectomy. To provide insights into wider application this technique, we performed a comprehensive literature review to appreciate the existing challenges and current status of minimally invasive donor hepatectomy.

A literature search was performed on PubMed (US National Library of Medicine, National Institutes of Health, United States) for relevant English articles with a combination of keywords: "LDLT" with "laparoscopy" and/or "laparoscopic assisted" and/or "hand assisted" and/or "subcostal incision" and/or "upper midline incision" and/or "robotic assisted". The references of the selected papers were reviewed for additional relevant articles.

DEFINITIONS

The minimally invasive approaches include reduced-wound (RW), pure laparoscopic (PL) and robotic-assisted (RA) procedures. In RW donor hepatectomy, resection is facilitated by a mini-laparotomy incision. RW approaches comprise hand-assisted laparoscopy[3], where resection is effected through laparoscopy but expedited by a hand port; the laparoscopic-assisted or hybrid approach[3], where laparoscopic mobilization (with or without hand assistance) is followed by open parenchymal transection; and mini-laparotomy, where resection is performed with an open technique via a reduced-length upper midline wound. In PL donor hepatectomy, liver resection is completed through laparoscopic ports. An auxiliary incision, usually suprapubic, is used only for graft retrieval. When a robotic system is involved, the procedure is considered an RA donor hepatectomy.

CHALLENGES

Limited role of LDLT in the West

LDLT expanded the donor pool and has become the predominant form of liver transplantation in the East due to the critical shortage of cadaveric donors. In the West, where deceased grafts are more widely available, LDLT is less desirable considering additional risks on the healthy live donor. In the United States, LDLT constitutes less than 5% of liver transplants[15]. None of the centres performed more than 30 live donor operations last year[15]. Limited volumes and experience have restricted the possibility of technical innovation. Although pioneered in Europe[12], minimally invasive donor hepatectomy has only been readily reproduced in Asia, where LDLT continued to flourish.

Albeit unpopular, LDLT continues to play a unique role in the West. When waitlist mortality is considered, recipients with access to a living donor have survival benefits[16]. In a cohort of patients listed for a liver graft in the United States, the risk of death of LDLT recipients was 50% less than those waiting for a cadaveric liver graft[17]. LDLT is most beneficial for transplant candidates with low priority to a cadaveric graft but at high risk of death while waiting for one[18]. These patients include those with low Model for End-stage Liver Disease scores but significant complications from portal hypertension, as well as patients with more advanced hepatocellular carcinoma, *i.e.*, at high risk to progress beyond criteria. In fact, with continuing efforts to foster live donation, the numbers of LDLTs have been growing in Canada[19]. Toronto has established the largest LDLT centre in the West, with LDLT accounting

for 30% of total liver transplants[20]. Their enthusiasm for promoting LDLT will promote ongoing technical advancements for the procedure.

Technical complexity

Laparoscopy revolutionized abdominal surgery, promoting the advantages of reduced morbidity and hastened recovery, and offering long-term outcomes comparable to those of open surgery[21-23]. While laparoscopy has become standard in gastric and colorectal surgery[22,23], its application in liver surgery has developed at a much slower pace. Complex vascular and biliary variations and potential major bleeding during parenchymal transection have made laparoscopic liver resection technically challenging. Reports indicate an average learning curve of 30-60 laparoscopic hepatectomies is required before operating time and blood loss can be optimized[24,25].

Donor hepatectomy entails additional technical demands. Precise transection of the bile duct is crucial to reduce biliary complications in both donor and recipient. Maintaining the correct parenchymal transection plane minimizes liver congestion and preserves graft function. Presence of vascular and biliary variations poses extra challenges. With respect to laparoscopic donor hepatectomy, parallel expertise in laparoscopic liver surgery and donor hepatectomy are required[26]. Laparoscopic left lateral sectionectomy, when performed for liver donation, requires approximately 20 procedures for an experienced transplant surgeon to achieve optimized blood loss and warm ischaemic time[27,28]. A precipitous learning curve is encountered before one can perpetuate proficiency in this complex procedure.

Donor safety

Donor safety is paramount in LDLT. Donor hepatectomy imposes 0.1%-0.2% mortality[29] and 25%-35% morbidity[1,2] to an healthy individual. As such, safety has been the primary obstacle to a wider application of minimally invasive approaches in the donor arena. During the early development of laparoscopic hepatectomy, management of venous haemorrhage during hepatic transection has been particularly problematic. High-flow venous tributaries within the liver parenchyma make laparoscopic transection technically challenging. However, through accumulation of experience, technical refinements have paved the way for safer approaches to liver resection. Surprisingly, lower blood loss has been achieved with laparoscopic hepatectomy[5-11], thanks to improved visualization and positive pressure from the pneumoperitoneum.

Biliary complications occur after 10% of donor hepatectomies[1], the majority of which require intervention. The most common site of a bile leak is the transection surface, from caudate branches or from the hilar plate[30]. Parenchymal transection in laparoscopic hepatectomy is expedited with energy devices, while small bile duct tributaries are usually controlled with clips instead of ligatures. The initial concern for more bile leaks after laparoscopic hepatectomy was unfounded after a meta-analysis revealed a lower leak rate of less than 2% in minimally invasive hepatectomy[4]. It is believed that laparoscopic magnification provides superior visibility for identifying tributary branches and minor leakages.

Meanwhile, laparoscopic management of bile duct division remains a topic of debate. Determining the site of bile duct transection is a unique and critical step in donor hepatectomy. Dividing too close to graft produces multiple bile duct openings, while keeping too flush to donor poses risk of biliary stricture. Presence of anatomical variations imposes additional technical challenges. An aberrant right hepatic duct occurs in 15% of the population, and 6% of the right posterior duct drains into left hepatic duct[31]. In the setting of donor hepatectomy, laparoscopy has to prove at least equivalent performance in managing bile duct transection before its application can be expanded.

Recipient outcomes

In PL and RA donor hepatectomy, the liver graft is retrieved through a small wound after enveloping in a plastic bag. The initial fear of a longer warm ischaemic time and its undesirable consequences has deprived acceptance for more innovative approaches. From the reported series, the donor warm ischaemic time varied from 3-12 min for PL approaches[13,32-37] to 8-15 min for RA approaches[38,39], which were not prolonged when compared with open procurement. More importantly, initial experiences in from left lateral sectionectomy showed that graft survivals were not different from the open approach[40].

PRESENT STATUS

Reduced wound donor hepatectomy

In the first decade of this century, application of PL donor hepatectomy was greatly limited by technical difficulties. Transplant surgeons refrained from PL donor hepatectomy in fear of damaging vital vascular pedicles and potential catastrophic bleeding. Alternative strategies were developed to reduce wound length while retaining the reliability of conventional hepatectomy. In the hand-assisted technique, a hand port allowed for versatile liver traction to facilitate exposure and haemostasis during transection[41]. Two hand-assisted right lobe donor hepatectomies were reported in a small series[42]. Reduced wound was more often utilized, as in the hybrid technique[43], where parenchymal transection was performed as an open procedure, after the liver was laparoscopically mobilized then retracted into the upper midline wound. The need for subcostal incision was avoided, while the safety of open transection was preserved. The hybrid technique gained popularity with multiple series reported for both right[42-52] and left lobe donation[44,47,49,52]. Over 200 hybrid donor hepatectomies have been performed worldwide with zero mortality and morbidities at least comparable to those of conventional

open surgery.

Transplant surgeons' passion for minimally invasive donor hepatectomy has not been limited by laparoscopy. Experienced centres advocated open right lobe donation through a 10-14 cm upper midline wound without laparoscopic assistance[46,53-56]. This mini-laparotomy approach represents a philosophy distinct from that of minimally invasive surgery. Laparoscopy provides improved visualization and laparoscopic instruments minimize tissue manipulation, both of which contribute to the potential benefits of minimally invasive surgery. Mini-laparotomy is the pure pursuit of wound reduction while preserving the essence of open surgery. The technique became the standard practice in high-volume LDLT centres in South Korea[54].

Donor and recipient outcomes of hybrid and mini-laparotomy approaches are summarized in Tables 1 and 2, respectively. Although inconsistently reported in case series, the benefits of reduced blood loss, wound pain and overall morbidity have been concluded in a meta-analysis comparing RW donor hepatectomy and open donor hepatectomy[57]. Types of complications were not specified. Neither was there a clarification of different types of RW donor hepatectomy. As hybrid and mini-laparotomy represented distinct approaches towards minimally invasive surgery, it is appealing to investigate whether the benefit of RW donor hepatectomy is a result of improved visualization or reduced abdominal wall trauma or a combination of the two.

Another meta-analysis by Berardi *et al*[58] might provide information regarding the performance of hybrid donor hepatectomy. The minimally invasive donor hepatectomy group in the study comprised mostly hybrid left or right donor hepatectomy (*n* = 227, 89%) and a few pure laparoscopic left lateral sectionectomies (*n* = 27, 11%). No mini-laparotomy patients were included. Based on the pooled data, hybrid hepatectomy and PL donor hepatectomy were associated with fewer wound-related (OR = 0.41, *P* = 0.04) but similar biliary complications when compared with open donor hepatectomy. Reduction in analgesia requirement (MD = -0.54, *P* = 0.04) and hospital stay (MD = -1.6, *P* = 0.004) was observed. Hybrid donor hepatectomies have validated its safety and potential benefits to the donor. This technique allows transplant surgeons to accumulate experience before converting to pure laparoscopic approaches. The only question that remains is likely that of long-term graft outcomes. Nevertheless, the contributions of hybrid donor hepatectomy to the evolution of minimally invasive donor hepatectomy cannot be overemphasized.

Pure laparoscopic donor Hepatectomy

Left lateral sectionectomy: Laparoscopic approaches to donor hepatectomy become least controversial with respect to left lateral section donation. The Falciform ligament, where the vertical portion of the left portal vein is situated, provides a well-defined surface landmark for left lateral sectionectomy[59]. A transection plane along its right side exposes the hilar plate for left portal vein and bile duct transection. The constant anatomy and a small parenchymal transection surface offer technical advantages. Indeed, left lateral section was the first living donor liver graft harvested conventionally[60] and laparoscopically[12].

Since its feasibility was reported in 2002[12], PL donor left lateral sectionectomy has been validated subsequently in several centres[28,40,61-64]. The results of these studies are summarized in Table 3. According to case-control studies, the PL approach is associated with reduced blood loss, shortened length of stay and comparable donor morbidity over open surgery[28,40,61]. To date, over 120 PL donor left lateral sectionectomies have been performed throughout the world[63], and the approach is regarded as the standard procedure in specialized centres. PL donor left lateral sectionectomy appears to be a safe and reproducible approach to LDLT.

Right hepatectomy: The right liver graft is the main form of LDLT providing adequate functional liver to the recipient[65]. The first PL donor right hepatectomy represented another quantum leap for minimally invasive donor hepatectomy. The procedure was first reported by Soubrane *et al*[13] in 2013, followed by several small-volume case series[32-37,66,67] (Table 4). The pioneering surgeons' achievement had not been readily reproduced until a larger series became available earlier this year[37].

Suh *et al*'s series of 45 PL donor right hepatectomies derived from the work of a single surgeon, who had tremendous experience encompassing over 1000 open donor hepatectomies as well as 200 laparoscopic hepatectomies. In the early phase, donors with single right portal vein and right hepatic ducts were selected. After sufficient experience, additional selection criteria were no longer applied, and the PL approach was performed in 90% of right lobe donors in the later phase. Biliary imaging was a combination of preoperative magnetic resonance cholangiopancreatography and intraoperative indocyanine green (ICG) cholangiography, abbreviating the need for conventional operative cholangiogram. Compared with historical controls who had undergone open right lobe donation by the same surgeon, PL donor right hepatectomy took longer (331 ± 50 min *vs* 280 ± 40 min, *P* < 0.001), had more blood loss (436 ± 170 mL *vs* 338 ± 188 mL, *P* = 0.013) and longer warm ischaemic time (12.6 ± 4.4 mL *vs* 5.4 ± 3.6 mL, *P* < 0.001). Incidences of donor (8.9% *vs* 11.9%, *P* = 0.73) and recipient complications (24.4% *vs* 26.2%, *P* = 0.85) were similar.

Notably, the PL approach produced more liver grafts with multiple bile duct openings (53% *vs* 26%, *P* < 0.001). The surgeon might err on the safe side to divide the bile duct close to the graft side. However, more bile duct openings made recipient biliary anastomosis more challenging, potentially compromising this outcome. In this series, donor bile duct was initially closed with intra-

Table 1 Outcomes of hand-assisted and laparoscopic-assisted donor hepatectomy

	LLS	Left	Right	OT (min)	Blood loss (mL)	WIT (min)	HS (d)	Donor Cx	Recipient Cx
Hand-assisted									
Suh et al[42], 2009			2	765-898	-	-	10-14	2 (100%)[a]	2 (100%)[b]
Hybrid									
Comparative study									
Kurosaki et al[44], 2006[c]	10/12	3/1		363 ± 33/320 ± 68	302 ± 191/283 ± 371	3	11.0 ± 2.7/12.8 ± 4.9	-	[d]
Baker et al[45], 2009			33/33	265 ± 58[1]/316 ± 61	417 ± 217/550 ± 305	-	4.3/3.9	7 (21.2%)[e]	-
Thenappan et al[46], 2011[f]	8/7			312 ± 68/324 ± 106	1033 ± 1096/733 ± 457	-	6.0 ± 2.0/6.4 ± 3.7	2 (13.3%)[g]	7 (46.7%)[h]
Choi et al[48], 2012[i]			40/20/90	279 ± 72/384 ± 42[1]/303 ± 61	450 ± 316/870 ± 653[1]/532 ± 323	-	11.8 ± 4.5/12.1 ± 2.8/12.0 ± 3.6	5 (12.5%)[j]/6 (30.0%)[k]	-
Marubashi et al[49], 2013[l]	17/32	14/47		435 ± 103[1]/383 ± 73	353 ± 396/456 ± 347		10.3 ± 3.3[1]/18.3 ± 16.7	3 (9.7%)[m]	-
Nagai et al[55], 2012[n]			19/30	371 ± 52/363 ± 53	212 ± 114[1]/316 ± 121	-	5.9 ± 1.2[1]/7.8 ± 2.3	7 (25.0%)[o]	10 (35.7%)[p]
Makki et al[50], 2014			26/24	703 ± 124/675 ± 118	337 ± 89/396 ± 126	-	-	4 (15.4%)[q]	2 (7.8%)[r]
Shen et al[51], 2016[s]			28/20	386 ± 49/366 ± 45	384 ± 180/416 ± 164	3.0 ± 1.6/2.9 ± 1.5	7.4 ± 2.5/7.3 ± 1.6	5 (17.9%)[t]	-
Kitajima et al[52], 2017	35/38			459 (310-633)[1]/403 (256-597)	245 (22-1840)/400 (20-1638)	-	12 (7-50)/12 (8-31)	8 (22.9%)[u]	13 (17.1%)[v]
			41/39	431 (310-651)[1]/402 (315-588)	201 (10-1559)[1]/313 (55-2165)	-	12 (8-27)/ 12 (7-40)	9 (22.0%)[w]	
Case series									
Koffron et al[43], 2006			1	235	150	-	3	0	0
Suh et al[42], 2009			7	310-575	-	-	8-17	4 (57.1%)[x]	5 (71.4%)[y]
Soyama et al[47], 2012		9	6	456 (328-581)	520 (230-1000)	-	-	1 (6.7%)[z]	-

[1]Statistically significant; [a]Intra-abdominal collection and pleural effusion in both patients; [b]Biliary stricture and stroke in one patient and bile leak and biliary stricture in the other patient; [c]Combined results of left and right hepatectomy; [d]Three (23%) early graft loss within 2 mo; [e]Small bowel injury (*n* = 1), biloma (*n* = 1) and other complications (*n* = 3); [f]Combined results of 15 hybrid and mini-laparotomy compared with 15 open operations, types of graft other than left lateral section not specified; [g]Bile leak (*n* = 1) and incisional hernia (*n* = 1); [h]Bile leak (*n* = 2), vascular complications (*n* = 3), intra-abdominal collection (*n* = 1) and chylous ascites (*n* = 1); [i]Single-port laparoscopic-assisted (*n* = 40) *vs* laparoscopic-assisted (*n* = 20) *vs* open (*n* = 90); [j]Intra-abdominal bleeding (*n* = 2), bile leak (*n* = 3) and pleural effusion (*n* = 1); [k]Wound complication (*n* = 2), diaphragmatic hernia (*n* = 1), pleural effusion (*n* = 2) and biliary stricture (*n* = 1); [l]Combined results of donor left lateral sectionectomy and left hepatectomy; [m]Delayed gastric emptying requiring endoscopy (*n* = 2) and grade I complication (*n* = 1); [n]Combined results of 19 hybrid and 9 mini-laparotomies compared with 30 open operations; [o]Intra-abdominal bleeding (*n* = 1), bile leak (*n* = 1), intra-abdominal collection (*n* = 1), ileus (*n* = 2), deep vein thrombosis (*n* = 1) and phlebitis (*n* = 1); [p]Bile leak (*n* = 2), biliary stricture (*n* = 2), hepatic artery stricture (*n* = 2), hepatic vein stricture (*n* = 2) and intra-abdominal collection (*n* = 2); [q]Pleural effusion requiring tapping (*n* = 1) and grade I complications (*n* = 3); [r]Bile leak (*n* = 1) and biliary stricture (*n* = 1); [s]Laparoscopic-assisted (*n* = 28) compared against mini-laparotomy (*n* = 20); [t]Intra-abdominal bleeding (*n* = 1), ileus (*n* = 1), pneumonia (*n* = 1) and pleural effusion (*n* = 2); [u]Bile leak (*n* = 3), intra-abdominal collection (*n* = 1), pneumonia (*n* = 1) and grade I complications (*n* = 3); [v]Combined results of left and right hepatectomy; bile leak (*n* = 5), biliary stricture (*n* = 5), portal vein thrombosis (*n* = 2), arterial complication (*n* = 1); [w]Fever of unknown origin (*n* = 2), renal failure (*n* = 1), small bowel obstruction (*n* = 1) and grade I complications (*n* = 5); [x]Bile leak (*n* = 1), intra-abdominal collection (*n* = 1) and pleural effusion (*n* = 3); [y]Bile leak (*n* = 1), portal vein thrombosis (*n* = 1) and biliary stricture (*n* = 3); [z]Portal vein thrombosis. Cx: Complications; HS: Hospital stay; LLS: Left lateral section; OT: Operating time; WIT: Warm ischaemic time.

corporeal suturing. After a bile leak was encountered, suturing was replaced by applying two metal clips on the donor side, which might have shifted the division point to the graft side. Nevertheless, recipient biliary complications were kept minimal (*n* = 1, 2.2%), reflecting the technical excellence of the implant surgeon. The occurrence of one hepatic artery thrombosis and two intra-operatively detected intimal dissections prompted the surgeon of a potential problem for PL donor hepatectomy. The author attributed the issue to intimal damage during intra-corporeal ligation (reduced tactile feedback) and retraction during caudate transection. From this series, it was concluded that PL donor right hepatectomy was a feasible procedure for experienced transplant surgeons. However, further evaluation is needed to standardize the techniques for better operative outcomes.

Left hepatectomy: Although a right liver graft is usually preferred for higher graft volume, donor right hepatectomy is associated with more morbidity than is left hepatectomy[68-70]. Considering that donor risk is essentially related to the proportion of the liver resected, the left liver graft is selected when graft volumes are deemed adequate. The first PL donor left hepatectomy was performed in 2012[71], followed by a small series followed[37,40,71-73]; currently, approximately 20 cases have been reported in the literature (Table 5). There was no donor death or major complications. However, with limited experience, no conclusions can be arrived at, apart from the technical feasibility of this procedure in selected donors in expert hands.

Recipient safety remained the primary concern of using a left lobe graft[74]. Smaller grafts put the recipient

Table 2 Outcomes of donor hepatectomy with mini-laparotomy

	LLS	Left	Right	OT (min)	Blood loss (mL)	WIT (min)	HS (d)	Donor Cx	Recipient Cx
Comparative study									
Kim et al[53], 2009			23/23	232 ± 29[1]/269 ± 37	186 ± 59/218 ± 67	-	10 ± 3/12 ± 4	3 (13.0%)[a]	1 (4.3%)[b]
Thenappan et al[46], 2011[c]	8/7	-	-	312 ± 68/324 ± 106	1033 ± 1096/733 ± 457	-	6.0 ± 2.0/6.4 ± 3.7	2 (13.3%)[d]	7 (46.7%)[e]
Nagai et al[55], 2012[f]			9/30	371 ± 52/363 ± 53	212 ± 114[1]/316 ± 121	-	5.9 ± 1.2[1]/7.8 ± 2.3	7 (25.0%)[g]	10 (35.7%)[h]
Shen et al[51], 2016[i]			20/28	366 ± 45/386 ± 50	416 ± 164/383 ± 180	2.9 ± 1.5/3.0 ± 1.6	7.3 ± 1.6/7.4 ± 2.5	1 (5.0%)[j]	
Case series									
Lee et al[54], 2011			141	254 ± 47	352 ± 144	-	10 ± 3	25 (17.7%)[k]	51 (36.2%)[l]

[1]Statistically significant; [a]Intra-abdominal bleeding (*n* = 2) and pleural effusion (*n* = 1); [b]Bile leak requiring laparotomy (*n* = 1); [c]Combined results of 15 hybrid and mini-laparotomy compared with 15 open operations, types of graft other than left lateral section not specified; [d]Bile leak (n=1) and incisional hernia (*n* = 1); [e]Bile leak (*n* = 2), vascular complications (*n* = 3), intra-abdominal collection (*n* = 1) and chylous ascites (*n* = 1); [f]Combined results of 19 hybrid and 9 mini-laparotomies compared with 30 open operations; [g]Intra-abdominal bleeding (*n* = 1), bile leak (*n* = 1), intra-abdominal collection (*n* = 1), ileus (*n* = 2), deep vein thrombosis (*n* = 1) and phlebitis (*n* = 1); [h]Bile leak (*n* = 2), biliary stricture (*n* = 2), hepatic artery stricture (*n* = 2), hepatic vein stricture (*n* = 2) and intra-abdominal collection (*n* = 2); [i]Mini-laparotomy (*n* = 20) compared against laparoscopic-assisted (*n* = 28); [j]Pneumonia; [k]Rhabdomyolysis (*n* = 1), intra-abdominal bleeding (*n* = 4), bile leak (*n* = 4), ileus (*n* = 2) and grade I complications (*n* = 14); [l]Biliary complications (*n* = 36), intra-abdominal bleeding (*n* = 5) and vascular complications (*n* = 6). Cx: Complications; HS: Hospital stay; LLS: Left lateral section; OT: Operating time; WIT: Warm ischaemic time.

Table 3 Outcomes of pure laparoscopic donor left lateral sectionectomy

	No.	OT (min)	Blood loss (mL)	WIT (min)	Conversion	HS (d)	Donor Cx	Recipient Cx
Comparative study								
Soubrane et al[28], 2006	16/14	320 ± 67[1]/224 ± 15	19 ± 44[1]/99 ± 185	10 (6-12)/5(2-7)	1 (6.3%)	7.5 ± 2.3/8.1 ± 3.0	3 (18.7%)[a]/5 (35.7%)	6 (37.5%)[b]/6 (42.8%)
Kim et al[61], 2011	11/11	330 ± 68/306 ± 29	396 ± 72/464 ± 78	6 ± 2/5 ± 1	0	6.9 ± 0.3[1]/9.8 ± 0.9	0/1 (9.1%)	2 (18.1%)[c]/2 (18.1%)
Samstein et al[40], 2015[d]	17/20	478 ± 68[1]/398 ± 42	177 ± 101[1]/375 ± 191	-	0	4.3 ± 1.5[1]/6.0 ± 1.5	2 (9.1%)[e]/5 (25%)	1 (4.5%)[f]/1 (4.5%)
Case series								
Cherqui et al[12], 2012	2	360-420	150-450	4-10	0	5-7	0	1 (50.0%)[g]
Yu et al[92], 2012	15	331 ± 63	410 ± 71	6 ± 2	0	7.1 ± 0.8	0	-
Scatton et al[62], 2015[h]	67	275 (175-520)	82 ± 79	9 ± 4	4 (5.7%)	6 (3-18)	17 (25.3%)[i]	
Soubrane et al[63], 2015[j]	124	308 (180-555)	50 (10-500)	8	5 (4.0%)	6.3 (2-18)	21 (16.9%)[k]	-
Troisi et al[64], 2017	11	237 ± 99	70 ± 41	4	0%	4	2 (18.1%)[l]	5 (45.4%)[m]

[1]Statistically significant; [a]Bile leak requiring laparoscopy (*n* = 1) and wound haematoma (*n* = 2); [b]Portal vein thrombosis requiring re-transplant (*n* = 1), hepatic artery thrombosis (*n* = 2) and biliary stricture (*n* = 3); [c]Portal vein stenosis requiring stenting (*n* = 1) and biliary stricture (*n* = 1); [d]Results included 5 left hepatectomy and compared with mixed open and hybrid controls; [e]Hernia (*n* = 1) and bile leak (*n* = 1); [f]Portal vein thrombosis requiring exploration (*n* = 1); [g]Hepatic artery thrombosis (*n* = 1); [h]Results included 3 left hepatectomy; [i]Bile leak (*n* = 2), biliary stricture (*n* = 1), pulmonary complications (*n* = 2), bladder injury (*n* = 1), and complications (*n* = 5); [j]Combined results of 5 centres; [k]Bile leak (*n* = 3), wound haematoma requiring drainage (*n* = 1), bladder injury requiring cystoscopy (*n* = 1), fluid collection requiring drainage (*n* = 1), others: grade I-II complications; [l]Hepatic necrosis (*n* = 1) and collection (*n* = 1) [m]Fungemia leading to death (*n* = 1) and biliary stricture (*n* = 4). Cx: Complications; HS: Hospital stay; LLS: Left lateral section; OT: Operating time; WIT: Warm ischaemic time.

at risk of small-for-size syndrome. Even when implanted with similarly sized grafts, left lobe recipients experienced more arduous recovery[75]. In a sense, the current status of PL donor left hepatectomy is primarily limited by the inherent disadvantage of the graft type. However, with time and experience, the undesirable consequences of small-for-size liver grafts have been minimized with refined surgical techniques[76-78]. A Japanese series of 200 left lobe recipients revealed long-term survivals comparable to those of right lobe recipients[79]. This re-emphasizes left lobe LDLT as a valuable option for LDLT, especially when donor remnant volume is marginal for right lobe donation.

Robotic-assisted donor hepatectomy
Interestingly, robotic surgery has taken the lead over laparoscopy regarding donor right hepatectomy. The first RA donor right hepatectomy was reported in 2012[38], one year before the first PL approach to this surgery[13]. Robotic systems offer a stable magnified field and

Table 4 Outcomes of pure laparoscopic donor right hepatectomy

	No.	OT (min)	Blood loss (mL)	WIT (min)	Conversion	HS (d)	Donor Cx	Recipient Cx
Comparative study								
Takahara *et al*[67], 2015	5/25	480 ± 54[1]/380 ± 45	91 ± 69[1]/268 ± 194	9	0	9.4 ± 1.8/9.0 ± 2.2	1 (20%)[a]	-
Suh *et al*[37], 2018	45/42	331 ± 50[1]/280 ± 40	436 ± 170[1]/338 ± 188	12.6 ± 4.4[1]/5.4 ± 3.6	0	8.2 ± 1.3/8.4 ± 1.0	5 (11.9%)[b]	11 (26.2%)[c]
Case series								
Soubrane *et al*[13], 2013	1	480	100	12	0	7	0	0
Rotellar *et al*[32], 2013	1	480	100	3	0	4	0	1 (100%)[d]
Han *et al*[33], 2015[e]	2	-	-	-	9	9 (8-10)	-	-
Chen *et al*[39], 2015	1	415	150	6	0	6	1 (100%)[f]	1 (100%)[g]
Kim *et al*[36], 2017	3	427-502	200-270	4.5-5.0	0	7-8	0	0

[1]Statistically significant; [a]Biliary complication; [b]Liver abscess (*n* = 1), Pneumonia (*n* = 1), upper respiratory tract infection (*n* = 1) and grade I complications (*n* = 2); [c]Intra-abdominal bleeding (*n* = 4), vascular complication (*n* = 4), biliary complication (*n* = 2) and others; [d]Pneumonia; [e]Video presentation; [f]Wound haematoma; [g]Pneumonia. Cx: Complications; HS: Hospital stay; OT: Operating time; WIT: Warm ischaemic time.

Table 5 Outcomes of pure laparoscopic donor left hepatectomy

	No.	OT (min)	Blood loss (mL)	WIT (min)	Conversion	HS (d)	Donor Cx	Recipient Cx
Comparative study								
Samstein *et al*[40], 2015[a]	5/20	478 ± 68[1]/398 ± 42	177 ± 101[1]/375 ± 191	-	0	4.3 ± 1.5[1]/6.0 ± 1.5	2 (9.1%)[b]/5 (25%)	1 (4.5%)[c]/1 (4.5%)
Case series								
Samstein *et al*[71], 2013	2	358-379	125	-	0	4 ± 1	0	1 (50%)[d]
Troisi *et al*[72], 2013	4	370-560	50-80	4-7	0	4-6	0	1 (25%)e
Almodhaiberi *et al*[73], 2018	1	300	125	-	0	8	0	-

[1]Statistically significant; [a]Results included 17 left lateral sectionectomies and compared with mixed open and hybrid controls; [b]Hernia (*n* = 1) and bile leak (*n* = 1); [c]Portal vein thrombosis requiring exploration (*n* = 1); [d]Bile leak (*n* = 1); [e]Recipient common hepatic artery dissection (*n* = 1). Cx: Complications; HS: Hospital stay; OT: Operating time; WIT: Warm ischaemic time.

provide ergonomic advantages beyond conventional laparoscopy, namely, improved range of motion and enhanced precision[80]. Articulated instruments allow for proper plications of venous bleeding. In the setting of donor hepatectomy, robotic system facilitates closure of the hepatic duct stump with a running suture[81]. Compared with clipping, suture closure requires a shorter bile duct length and potentially reduces the probability of multiple graft bile duct openings or donor biliary strictures.

RA donor right hepatectomy was reproduced in a series reported by Chen *et al*[39] comparing 13 RA against 54 open procedures. The operating time in the RA group (596 min) was prolonged even when relative to that of PL approaches reported in the literature[13,32-37]. Nevertheless, warm ischaemic time (10 min) did not appear to be an issue for graft retrieval with a robotic system. Compared with open hepatectomy, RA procedures had similar blood loss (169 mL *vs* 146 mL, *P* = 0.47) and overall morbidities (7.7% *vs* 9.3%, *P* = 0.68). With respect to donor benefits, reduction in analgesia (PCA/BW on D1 0.58 ng/kg *vs* 0.84 ng/kg, *P* = 0.03) and shorter returns to work (52.9 d *vs* 100 d, *P* = 0.02) and sex (100 d *vs* 156 d, *P* = 0.047) were reported. In the recipients, incidences of vascular and biliary complications were similar and liver functions

were comparable upon 1-year follow-up. With promising early results, the remaining issue is likely an exceedingly protracted learning curve. Expertise in robotic procedures is desired in addition to proficiency in laparoscopic hepatobiliary surgery and donor hepatectomy.

ARE WE READY FOR PRIME TIME?

Upon reviewing the literature, the benefits of PL approaches have been validated for more simple procedures in the case of left lateral sectionectomy. For lobar liver donation, technical feasibility has been demonstrated by experienced surgeons, yet reproducibility is likely limited by the precipitous learning curve as well as safety concerns. Limited evidence supports the potential advantages of adopting a PL approach. The subsequent section discusses strategies for overcoming these obstacles and ensuring a safe transition to minimally invasive donor hepatectomy.

Donor selection

The importance of cautious donor selection was demonstrated in Kim *et al*[36]'s report on PL donor right hepatectomy. In the authors' series, 3 donors were selected among 92 candidates (4%), from a centre with tremendous experience encompassing over 3500 LDLT

operations. Strict selection criteria were applied, with emphasis on vascular and biliary anatomy. Donors with single and longer right hepatic artery, right portal vein and right hepatic duct were selected. The authors also excluded donors with larger estimated grafts, *i.e.*, more than 650 g. Similar criteria were applied in the early phase of Suh *et al*[37]'s series. Favourable anatomy allows for the acquisition of experience and standardization of techniques before more challenging anatomy can be safely handled. However, biliary variation per se should not be considered a contraindication to PL approaches, given the availability of surgical expertise. In fact, successful laparoscopic management of complicated biliary anatomy has been reported with no donor or recipient morbidity[34,66].

Technical standardization

Technical standardization may be the key to improving the safety and reproducibility of complex and sophisticated procedures. Based on experience in oncological liver resections, several basic skills are essential to laparoscopic liver resection[82]. Liver resection is preceded by complete mobilization so that transection plane can be manipulated. After hilar dissection, the Glissonian pedicle is encircled. Surface parenchyma up to a depth of 2 cm is transected with energy devices, as there are no vital hepatic pedicles within superficial parenchyma. Deep parenchymal transection is effected through a Cavitron Ultrasonic Surgical Aspirator (CUSA™, Tyco Healthcare, Mansfield, MA, United States) because it is important not to damage intra-parenchymal hepatic structures. Small tributaries at the transection surface are controlled with a combination of clips and bipolar forceps.

The hanging manoeuvre has been demonstrated to be highly effective in open liver resections[83]. Passage of a cotton tape along the avascular plane between the liver and the inferior vena cava allows for the liver to be suspended posteriorly. This manoeuvre reduces venous bleeding and guides transection along Cantlie's line. The lateral approach is a modification of this technique for laparoscopy[84]. Instead of developing the avascular plane, the hanging tape is placed lateral to the inferior vena cava for right hepatectomy or between the inferior vena cava and ligamentum venosum for left hepatectomy. The need for dissection of the avascular plane, and hence the problematic bleeding from caudate branches, was abbreviated. This technique is simple, effective and applicable to different approaches of minimally invasive donor hepatectomy.

Intermittent inflow control with Pringle's manoeuvre[85] also reduces blood loss during hepatic transection, but its use in the setting of LDLT is controversial. While detractors have raised the concern of potential ischaemic graft injury, routine intermittent inflow control has been adopted in several transplant units[86-88]. In a randomized controlled trial. inflow control was performed with intermittent 15 min clamping and 5 min release cycles. The results confirmed no increase in recipient alanine

aminotransferase (peak 477 U/mL *vs* 345 U/mL, *P* = 0.32) or international normalized ratio (peak 2.6 *vs* 2.5, *P* = 0.44), while the donor blood loss was reduced (324 mL *vs* 486 mL, *P* = 0.02)[88]. With evidence validating its safety, inflow occlusion remains an optional manoeuvre in LDLT without compromising graft function.

In donor hepatectomy, operative cholangiogram is essential to determining the site of bile duct division. Operative cholangiogram is usually performed after surgeons leave the surgical field. In the PL or RA approach, surgeons can remain the operative position during fluoroscopy[32]. Real-time fluoroscopic guidance enhances precision and safety of bile duct division. Fluorescence imaging with ICG is a novel technique for intraoperative cholangiogram[89]. ICG can be injected intravenously or directly into the biliary tree *via* the cystic duct stump[90]. Intravenous ICG injection is the preferred technique given its simplicity. Instead of producing a separate plain image, the fluorescence of ICG is completely incorporated into the laparoscopic view. This approach provides real-time navigation with greatly enhanced accuracy. The largest series of RA donor right hepatectomy was performed with intravenous ICG cholangiography[39]. One inherent limitation of ICG cholangiography is that the biliary tree can only be imaged when adequately exposed. An aberrant duct situated deeply in hepatic parenchyma may not be readily imaged. Perhaps a more effective approach is a combination of the two techniques. While a conventional cholangiogram remains essential to imaging any anatomical variation, fluorescence cholangiography might add precision in fine tuning the division point. In addition, ICG injection after temporary control of portal pedicles enhances visualization of ischaemic demarcation. Precise dissection along Cantle's line minimizes blood loss and avoids leaving ischaemic parenchyma to graft and donor.

Prospective registry

Current studies on minimally invasive donor hepatectomies are primarily retrospective case control studies or case series, which can be limited by selection bias. With regard to donor safety, a prospective study with preoperative enrolment may be a better option. In donor operations, severe complications are the major concern. Due to limited sampling, uncommon but sinister complications may not be readily detected by a randomized controlled trial. In this setting, a prospective registry is an effective alternative. When laparoscopic cholecystectomy was introduced, bile duct injuries were more readily detected by a prospective registry than by randomized controlled trials[91]. The Louisville statement emphasized the importance of a prospective registry to evaluate the safety of laparoscopic hepatectomy[3]. As PL donor hepatectomy has not been evaluated in a randomized control trial. which can be logistically difficult, an international prospective registry can be initiated. Broad participation from transplant centres with available

expertise is encouraged so that the safety of donor procedures can be effectively evaluated.

CONCLUSION

Despite critics and challenges, minimally invasive donor hepatectomy has been performed with increasing frequency. Donor left lateral sectionectomy has provided most anatomical advantages for pure laparoscopic surgery. The technique has been well validated for its safety and advantages and has become the standard in experienced centres[63]. RW donor hepatectomy, either in the form of a laparoscopic-assisted technique or utilizing a mini-laparotomy wound, has guided surgeons' transition from open donor hepatectomy to PL approaches. With accumulation of experience, PL donor right hepatectomy has been shown to be technically feasible. RA donor hepatectomy also appears to be a valuable alternative to PL donor hepatectomy.

Existing reports were derived from centres with tremendous experience in both laparoscopic hepatectomy and donor hepatectomy. The technical complexity associated with these procedures indicates an arduous transition from technical feasibility to reproducibility and disseminated application. Creation of an international prospective registry is awaited to centralize expert input for assessing the relevance of this approach. Moreover, careful donor selection and adopting standardized techniques should allow transplant surgeons to acquire technical proficiency in this procedure. A cautious approach is crucial. as one untoward event in donor surgery may significantly set back progress. After all, the ongoing successful evolution of PL donor hepatectomy will ultimately depend on donor safety.

REFERENCES

1 **Ghobrial RM**, Freise CE, Trotter JF, Tong L, Ojo AO, Fair JH, Fisher RA, Emond JC, Koffron AJ, Pruett TL, Olthoff KM; A2ALL Study Group. Donor morbidity after living donation for liver transplantation. *Gastroenterology* 2008; **135**: 468-476 [PMID: 18505689 DOI: 10.1053/j.gastro.2008.04.018]

2 **Abecassis MM**, Fisher RA, Olthoff KM, Freise CE, Rodrigo DR, Samstein B, Kam I, Merion RM; A2ALL Study Group. Complications of living donor hepatic lobectomy--a comprehensive report. *Am J Transplant* 2012; **12**: 1208-1217 [PMID: 22335782 DOI: 10.1111/j.1600-6143.2011.03972.x]

3 **Buell JF**, Cherqui D, Geller DA, O'Rourke N, Iannitti D, Dagher I, Koffron AJ, Thomas M, Gayet B, Han HS, Wakabayashi G, Belli G, Kaneko H, Ker CG, Scatton O, Laurent A, Abdalla EK, Chaudhury P, Dutson E, Gamblin C, D'Angelica M, Nagorney D, Testa G, Labow D, Manas D, Poon RT, Nelson H, Martin R, Clary B, Pinson WC, Martinie J, Vauthey JN, Goldstein R, Roayaie S, Barlet D, Espat J, Abecassis M, Rees M, Fong Y, McMasters KM, Broelsch C, Busuttil R, Belghiti J, Strasberg S, Chari RS; World Consensus Conference on Laparoscopic Surgery. The international position on laparoscopic liver surgery: The Louisville Statement, 2008. *Ann Surg* 2009; **250**: 825-830 [PMID: 19916210]

4 **Nguyen KT**, Gamblin TC, Geller DA. World review of laparoscopic liver resection-2,804 patients. *Ann Surg* 2009; **250**: 831-841 [PMID: 19801936 DOI: 10.1097/SLA.0b013e3181b0c4df]

5 **Nguyen KT**, Marsh JW, Tsung A, Steel JJ, Gamblin TC, Geller DA. Comparative benefits of laparoscopic vs open hepatic resection: a critical appraisal. *Arch Surg* 2011; **146**: 348-356 [PMID: 21079109 DOI: 10.1001/archsurg.2010.248]

6 **Simillis C**, Constantinides VA, Tekkis PP, Darzi A, Lovegrove R, Jiao L, Antoniou A. Laparoscopic versus open hepatic resections for benign and malignant neoplasms--a meta-analysis. *Surgery* 2007; **141**: 203-211 [PMID: 17263977 DOI: 10.1016/j.surg.2006.06.035]

7 **Croome KP**, Yamashita MH. Laparoscopic vs open hepatic resection for benign and malignant tumors: An updated meta-analysis. *Arch Surg* 2010; **145**: 1109-1118 [PMID: 21079101 DOI: 10.1001/archsurg.2010.227]

8 **Zhou YM**, Shao WY, Zhao YF, Xu DH, Li B. Meta-analysis of laparoscopic versus open resection for hepatocellular carcinoma. *Dig Dis Sci* 2011; **56**: 1937-1943 [PMID: 21259071 DOI: 10.1007/s10620-011-1572-7]

9 **Mirnezami R**, Mirnezami AH, Chandrakumaran K, Abu Hilal M, Pearce NW, Primrose JN, Sutcliffe RP. Short- and long-term outcomes after laparoscopic and open hepatic resection: systematic review and meta-analysis. *HPB* (Oxford) 2011; **13**: 295-308 [PMID: 21492329 DOI: 10.1111/j.1477-2574.2011.00295.x]

10 **Mizuguchi T**, Kawamoto M, Meguro M, Shibata T, Nakamura Y, Kimura Y, Furuhata T, Sonoda T, Hirata K. Laparoscopic hepatectomy: a systematic review, meta-analysis, and power analysis. *Surg Today* 2011; **41**: 39-47 [PMID: 21191689 DOI: 10.1007/s00595-010-4337-6]

11 **Li N**, Wu YR, Wu B, Lu MQ. Surgical and oncologic outcomes following laparoscopic versus open liver resection for hepatocellular carcinoma: A meta-analysis. *Hepatol Res* 2012; **42**: 51-59 [PMID: 21988222 DOI: 10.1111/j.1872-034X.2011.00890.x]

12 **Cherqui D**, Soubrane O, Husson E, Barshasz E, Vignaux O, Ghimouz M, Branchereau S, Chardot C, Gauthier F, Fagniez PL, Houssin D. Laparoscopic living donor hepatectomy for liver transplantation in children. *Lancet* 2002; **359**: 392-396 [PMID: 11844509 DOI: 10.1016/S0140-6736(02)07598-0]

13 **Soubrane O**, Perdigao Cotta F, Scatton O. Pure laparoscopic right hepatectomy in a living donor. *Am J Transplant* 2013; **13**: 2467-2471 [PMID: 23865716 DOI: 10.1111/ajt.12361]

14 **Hüscher CG**, Lirici MM, Chiodini S, Recher A. Current position of advanced laparoscopic surgery of the liver. *J R Coll Surg Edinb* 1997; **42**: 219-225 [PMID: 9276552]

15 OPTN: Organ Procurement and Transplantation Network - OPTN. Accessed March10, 2018 Available from: URL: https://optn.transplant.hrsa.gov

16 **Berg CL**, Merion RM, Shearon TH, Olthoff KM, Brown RS Jr, Baker TB, Everson GT, Hong JC, Terrault N, Hayashi PH, Fisher RA, Everhart JE. Liver transplant recipient survival benefit with living donation in the model for endstage liver disease allocation era. *Hepatology* 2011; **54**: 1313-1321 [PMID: 21688284 DOI: 10.1002/hep.24494]

17 **Maluf DG**, Stravitz RT, Cotterell AH, Posner MP, Nakatsuka M, Sterling RK, Luketic VA, Shiffman ML, Ham JM, Marcos A, Behnke MK, Fisher RA. Adult living donor versus deceased donor liver transplantation: a 6-year single center experience. *Am J Transplant* 2005; **5**: 149-156 [PMID: 15636624 DOI: 10.1111/j.1600-6143.2004.00654.x]

18 **Kim PT**, Testa G. Living donor liver transplantation in the USA. *Hepatobiliary Surg Nutr* 2016; **5**: 133-140 [PMID: 27115007 DOI: 10.3978/j.issn.2304-3881.2015.06.01]

19 **Levy GA**, Selzner N, Grant DR. Fostering liver living donor liver transplantation. *Curr Opin Organ Transplant* 2016; **21**: 224-230 [PMID: 26867047 DOI: 10.1097/MOT.0000000000000280]

20 **Sapisochin G**, Goldaracena N, Laurence JM, Levy GA, Grant DR, Cattral MS. Right lobe living-donor hepatectomy-the Toronto approach, tips and tricks. *Hepatobiliary Surg Nutr* 2016; **5**: 118-126 [PMID: 27115005 DOI: 10.3978/j.issn.2304-3881.2015.07.03]

21 **Reynolds W Jr**. The first laparoscopic cholecystectomy. *JSLS* 2001; **5**: 89-94 [PMID: 11304004]

22 **Kitano S**, Shiraishi N, Uyama I, Sugihara K, Tanigawa N;

Japanese Laparoscopic Surgery Study Group. A multicenter study on oncologic outcome of laparoscopic gastrectomy for early cancer in Japan. *Ann Surg* 2007; **245**: 68-72 [PMID: 17197967 DOI: 10.1097/01.sla.0000225364.03133.f8]

23 **Hazebroek EJ**; Color Study Group. COLOR: a randomized clinical trial comparing laparoscopic and open resection for colon cancer. *Surg Endosc* 2002; **16**: 949-953 [PMID: 12163961 DOI: 10.1007/s00464-001-8165-z]

24 **Vigano L**, Laurent A, Tayar C, Tomatis M, Ponti A, Cherqui D. The learning curve in laparoscopic liver resection: improved feasibility and reproducibility. *Ann Surg* 2009; **250**: 772-782 [PMID: 19801926 DOI: 10.1097/SLA.0b013e3181bd93b2]

25 **Cai X**, Li Z, Zhang Y, Yu H, Liang X, Jin R, Luo F. Laparoscopic liver resection and the learning curve: a 14-year, single-center experience. *Surg Endosc* 2014; **28**: 1334-1341 [PMID: 24399518 DOI: 10.1007/s00464-013-3333-5]

26 **Cauchy F**, Schwarz L, Scatton O, Soubrane O. Laparoscopic liver resection for living donation: where do we stand? *World J Gastroenterol* 2014; **20**: 15590-15598 [PMID: 25400442 DOI: 10.3748/wjg.v20.i42.15590]

27 **Troisi RI**. Open or laparoscopic living donor liver hepatectomy: still a challenging operation! *Am J Transplant* 2014; **14**: 736 [PMID: 24447688 DOI: 10.1111/ajt.12611]

28 **Soubrane O**, Cherqui D, Scatton O, Stenard F, Bernard D, Branchereau S, Martelli H, Gauthier F. Laparoscopic left lateral sectionectomy in living donors: safety and reproducibility of the technique in a single center. *Ann Surg* 2006; **244**: 815-820 [PMID: 17060776 DOI: 10.1097/01.sla.0000218059.31231.b6]

29 **Cheah YL**, Simpson MA, Pomposelli JJ, Pomfret EA. Incidence of death and potentially life-threatening near-miss events in living donor hepatic lobectomy: a world-wide survey. *Liver Transpl* 2013; **19**: 499-506 [PMID: 23172840 DOI: 10.1002/lt.23575]

30 **Kapoor S**, Nundy S. Bile duct leaks from the intrahepatic biliary tree: a review of its etiology, incidence, and management. *HPB Surg* 2012; **2012**: 752932 [PMID: 22645406 DOI: 10.1155/2012/752932]

31 **Mizumoto R**, Suzuki H. Surgical anatomy of the hepatic hilum with special reference to the caudate lobe. *World J Surg* 1988; **12**: 2-10 [PMID: 3344582]

32 **Rotellar F**, Pardo F, Benito A, Martí-Cruchaga P, Zozaya G, Lopez L, Hidalgo F, Sangro B, Herrero I. Totally laparoscopic right-lobe hepatectomy for adult living donor liver transplantation: useful strategies to enhance safety. *Am J Transplant* 2013; **13**: 3269-3273 [PMID: 24266975 DOI: 10.1111/ajt.12471]

33 **Han HS**, Cho JY, Yoon YS, Hwang DW, Kim YK, Shin HK, Lee W. Total laparoscopic living donor right hepatectomy. *Surg Endosc* 2015; **29**: 184 [PMID: 24993170 DOI: 10.1007/s00464-014-3649-9]

34 **Chen KH**, Huang CC, Siow TF, Chio UC, Chen SD, Chen YD, Lin TC, Huang SY, Wu JM, Jeng KS. Totally laparoscopic living donor right hepatectomy in a donor with trifurcation of bile duct. *Asian J Surg* 2016; **39**: 51-55 [PMID: 26211878 DOI: 10.1016/J.ASJSUR.2015.01.012]

35 **Takahara T**, Wakabayashi G, Nitta H, Hasegawa Y, Katagiri H, Umemura A, Takeda D, Makabe K, Otsuka K, Koeda K, Sasaki A. The First Comparative Study of the Perioperative Outcomes Between Pure Laparoscopic Donor Hepatectomy and Laparoscopy-Assisted Donor Hepatectomy in a Single Institution. *Transplantation* 2017; **101**: 1628-1636 [PMID: 28157736 DOI: 10.1097/TP.0000000000001675]

36 **Kim KH**, Kang SH, Jung DH, Yoon YI, Kim WJ, Shin MH, Lee SG. Initial Outcomes of Pure Laparoscopic Living Donor Right Hepatectomy in an Experienced Adult Living Donor Liver Transplant Center. *Transplantation* 2017; **101**: 1106-1110 [PMID: 28072754 DOI: 10.1097/TP.0000000000001637]

37 **Suh KS**, Hong SK, Lee KW, Yi NJ, Kim HS, Ahn SW, Yoon KC, Choi JY, Oh D, Kim H. Pure laparoscopic living donor hepatectomy: Focus on 55 donors undergoing right hepatectomy. *Am J Transplant* 2018; **18**: 434-443 [PMID: 28787763 DOI: 10.1111/ajt.14455]

38 **Giulianotti PC**, Tzvetanov I, Jeon H, Bianco F, Spaggiari M, Oberholzer J, Benedetti E. Robot-assisted right lobe donor hepatectomy. *Transpl Int* 2012; **25**: e5-e9 [PMID: 22029717 DOI: 10.1111/j.1432-2277.2011.01373.x]

39 **Chen PD**, Wu CY, Hu RH, Ho CM, Lee PH, Lai HS, Lin MT, Wu YM. Robotic liver donor right hepatectomy: A pure, minimally invasive approach. *Liver Transpl* 2016; **22**: 1509-1518 [PMID: 27509325 DOI: 10.1002/lt.24522]

40 **Samstein B**, Griesemer A, Cherqui D, Mansour T, Pisa J, Yegiants A, Fox AN, Guarrera JV, Kato T, Halazun KJ, Emond J. Fully laparoscopic left-sided donor hepatectomy is safe and associated with shorter hospital stay and earlier return to work: A comparative study. *Liver Transpl* 2015; **21**: 768-773 [PMID: 25789460 DOI: 10.1002/lt.24116]

41 **Fong Y**, Jarnagin W, Conlon KC, DeMatteo R, Dougherty E, Blumgart LH. Hand-assisted laparoscopic liver resection: lessons from an initial experience. *Arch Surg* 2000; **135**: 854-859 [PMID: 10896382 DOI: 10.1001/archsurg.135.7.854]

42 **Suh KS**, Yi NJ, Kim T, Kim J, Shin WY, Lee HW, Han HS, Lee KU. Laparoscopy-assisted donor right hepatectomy using a hand port system preserving the middle hepatic vein branches. *World J Surg* 2009; **33**: 526-533 [PMID: 19115031 DOI: 10.1007/s00268-008-9842-z]

43 **Koffron AJ**, Kung R, Baker T, Fryer J, Clark L, Abecassis M. Laparoscopic-assisted right lobe donor hepatectomy. *Am J Transplant* 2006; **6**: 2522-2525 [PMID: 16889605 DOI: 10.1111/j.1600-6143.2006.01498.x]

44 **Kurosaki I**, Yamamoto S, Kitami C, Yokoyama N, Nakatsuka H, Kobayashi T, Watanabe T, Oya H, Sato Y, Hatakeyama K. Video-assisted living donor hemihepatectomy through a 12-cm incision for adult-to-adult liver transplantation. *Surgery* 2006; **139**: 695-703 [PMID: 16701104 DOI: 10.1016/j.surg.2005.12.002]

45 **Baker TB**, Jay CL, Ladner DP, Preczewski LB, Clark L, Holl J, Abecassis MM. Laparoscopy-assisted and open living donor right hepatectomy: a comparative study of outcomes. *Surgery* 2009; **146**: 817-23; discussion 823-5 [PMID: 19789043 DOI: 10.1016/j.surg.2009.05.022]

46 **Thenappan A**, Jha RC, Fishbein T, Matsumoto C, Melancon JK, Girlanda R, Shetty K, Laurin J, Plotkin J, Johnson L. Liver allograft outcomes after laparoscopic-assisted and minimal access live donor hepatectomy for transplantation. *Am J Surg* 2011; **201**: 450-455 [PMID: 21421098 DOI: 10.1016/j.amjsurg.2010.10.007]

47 **Soyama A**, Takatsuki M, Hidaka M, Muraoka I, Tanaka T, Yamaguchi I, Kinoshita A, Hara T, Eguchi S. Standardized less invasive living donor hemihepatectomy using the hybrid method through a short upper midline incision. *Transplant Proc* 2012; **44**: 353-355 [PMID: 22410014 DOI: 10.1016/j.transproceed.2012.01.050]

48 **Choi HJ**, You YK, Na GH, Hong TH, Shetty GS, Kim DG. Single-port laparoscopy-assisted donor right hepatectomy in living donor liver transplantation: sensible approach or unnecessary hindrance? *Transplant Proc* 2012; **44**: 347-352 [PMID: 22410013 DOI: 10.1016/j.transproceed.2012.01.018]

49 **Marubashi S**, Wada H, Kawamoto K, Kobayashi S, Eguchi H, Doki Y, Mori M, Nagano H. Laparoscopy-assisted hybrid left-side donor hepatectomy. *World J Surg* 2013; **37**: 2202-2210 [PMID: 23736986 DOI: 10.1007/s00268-013-2117-3]

50 **Makki K**, Chorasiya VK, Sood G, Srivastava PK, Dargan P, Vij V. Laparoscopy-assisted hepatectomy versus conventional (open) hepatectomy for living donors: when you know better, you do better. *Liver Transpl* 2014; **20**: 1229-1236 [PMID: 24961992 DOI: 10.1002/lt.23940]

51 **Shen S**, Zhang W, Jiang L, Yan L, Yang J. Comparison of Upper Midline Incision With and Without Laparoscopic Assistance for Living-Donor Right Hepatectomy. *Transplant Proc* 2016; **48**: 2726-2731 [PMID: 27788808 DOI: 10.1016/j.transproceed.2016.03.046]

52 **Kitajima T**, Kaido T, Iida T, Seo S, Taura K, Fujimoto Y, Ogawa K, Hatano E, Okajima H, Uemoto S. Short-term outcomes of laparoscopy-assisted hybrid living donor hepatectomy:

a comparison with the conventional open procedure. *Surg Endosc* 2017; **31**: 5101-5110 [PMID: 28444493 DOI: 10.1007/s00464-017-5575-0]

53   **Kim SH**, Cho SY, Lee KW, Park SJ, Han SS. Upper midline incision for living donor right hepatectomy. *Liver Transpl* 2009; **15**: 193-198 [PMID: 19177437 DOI: 10.1002/lt.21677]

54   **Lee KW**, Kim SH, Han SS, Kim YK, Cho SY, You T, Park SJ. Use of an upper midline incision for living donor partial hepatectomy: a series of 143 consecutive cases. *Liver Transpl* 2011; **17**: 969-975 [PMID: 21584929 DOI: 10.1002/lt.22337]

55   **Nagai S**, Brown L, Yoshida A, Kim D, Kazimi M, Abouljoud MS. Mini-incision right hepatic lobectomy with or without laparoscopic assistance for living donor hepatectomy. *Liver Transpl* 2012; **18**: 1188-1197 [PMID: 22685084 DOI: 10.1002/lt.23488]

56   **Liu R**, Shen Y, Nan K, Mi B, Wu T, Guo J, Li M, Lv Y, Guo H. Association Between Expression of Cancer Stem Cell Markers and Poor Differentiation of Hepatocellular Carcinoma: A Meta-Analysis (PRISMA). *Medicine* (Baltimore) 2015; **94**: e1306 [PMID: 26252310 DOI: 10.1097/MD.0000000000001306]

57   **Zhang B**, Pan Y, Chen K, Maher H, Chen MY, Zhu HP, Zhu YB, Dai Y, Chen J, Cai XJ. Laparoscopy-Assisted versus Open Hepatectomy for Live Liver Donor: Systematic Review and Meta-Analysis. *Can J Gastroenterol Hepatol* 2017; **2017**: 2956749 [PMID: 29238704 DOI: 10.1155/2017/2956749]

58   **Berardi G**, Tomassini F, Troisi RI. Comparison between minimally invasive and open living donor hepatectomy: A systematic review and meta-analysis. *Liver Transpl* 2015; **21**: 738-752 [PMID: 25821097 DOI: 10.1002/lt.24119]

59   **Ma K,** Cheung T. Standardizing laparoscopic left lateral sectionectomy for hepatocellular carcinoma. In: Recent Advances in Hepatocellular Cancer. **2016**: 2-17. Accessed March 11, 2018. Available from: URL: https://hub.hku.hk/bitstream/10722/233418/1/Content.pdf

60   **Raia S**, Nery JR, Mies S. Liver transplantation from live donors. *Lancet* 1989; **2**: 497 [PMID: 2570198]

61   **Kim KH**, Jung DH, Park KM, Lee YJ, Kim DY, Kim KM, Lee SG. Comparison of open and laparoscopic live donor left lateral sectionectomy. *Br J Surg* 2011; **98**: 1302-1308 [PMID: 21717424 DOI: 10.1002/bjs.7601]

62   **Scatton O**, Katsanos G, Boillot O, Goumard C, Bernard D, Stenard F, Perdigao F, Soubrane O. Pure laparoscopic left lateral sectionectomy in living donors: from innovation to development in France. *Ann Surg* 2015; **261**: 506-512 [PMID: 24646560 DOI: 10.1097/SLA.0000000000000642]

63   **Soubrane O**, de Rougemont O, Kim KH, Samstein B, Mamode N, Boillot O, Troisi RI, Scatton O, Cauchy F, Lee SG, Griesemer A, Ahmed Z, Clavien PA, Cherqui D. Laparoscopic Living Donor Left Lateral Sectionectomy: A New Standard Practice for Donor Hepatectomy. *Ann Surg* 2015; **262**: 757-761; discussion 761-3 [PMID: 26583663 DOI: 10.1097/SLA.0000000000001485]

64   **Troisi RI**, Berardi G. Laparoscopic left lateral sectionectomy for living liver donation: the Ghent University experience. *Ann Laparosc Endosc Surg* 2017; **2**: 100 [DOI: 10.21037/ales.2017.04.09]

65   **Lo CM**, Fan ST, Liu CL, Wei WI, Lo RJ, Lai CL, Chan JK, Ng IO, Fung A, Wong J. Adult-to-adult living donor liver transplantation using extended right lobe grafts. *Ann Surg* 1997; **226**: 261-269; discussion 269-270 [PMID: 9339932]

66   **Han YS**, Ha H, Kwon HJ, Chun JM. Pure laparoscopic donor right hepatectomy in a living donor with type 3a biliary variation: A case report. *Medicine* (Baltimore) 2017; **96**: e8076 [PMID: 28930845 DOI: 10.1097/MD.0000000000008076]

67   **Takahara T**, Wakabayashi G, Hasegawa Y, Nitta H. Minimally invasive donor hepatectomy: evolution from hybrid to pure laparoscopic techniques. *Ann Surg* 2015; **261**: e3-e4 [PMID: 25185481 DOI: 10.1097/SLA.0000000000000898]

68   **Trotter JF**, Adam R, Lo CM, Kenison J. Documented deaths of hepatic lobe donors for living donor liver transplantation. *Liver Transpl* 2006; **12**: 1485-1488 [PMID: 16952175 DOI: 10.1002/lt.20875]

69   **Brown RS Jr**. Live donors in liver transplantation. *Gastroenterology* 2008; **134**: 1802-1813 [PMID: 18471556 DOI: 10.1053/j.gastro.2008.02.092]

70   **Taketomi A**, Kayashima H, Soejima Y, Yoshizumi T, Uchiyama H, Ikegami T, Yamashita Y, Harada N, Shimada M, Maehara Y. Donor risk in adult-to-adult living donor liver transplantation: impact of left lobe graft. *Transplantation* 2009; **87**: 445-450 [PMID: 19202452 DOI: 10.1097/TP.0b013e3181943d46]

71   **Samstein B**, Cherqui D, Rotellar F, Griesemer A, Halazun KJ, Kato T, Guarrera J, Emond JC. Totally laparoscopic full left hepatectomy for living donor liver transplantation in adolescents and adults. *Am J Transplant* 2013; **13**: 2462-2466 [PMID: 24034709 DOI: 10.1111/ajt.12360]

72   **Troisi RI**, Wojcicki M, Tomassini F, Houtmeyers P, Vanlander A, Berrevoet F, Smeets P, Van Vlierberghe H, Rogiers X. Pure laparoscopic full-left living donor hepatectomy for calculated small-for-size LDLT in adults: proof of concept. *Am J Transplant* 2013; **13**: 2472-2478 [PMID: 23914734 DOI: 10.1111/ajt.12362]

73   **Almodhaiberi H**, Kim SH, Kim KH. Totally laparoscopic living donor left hepatectomy for liver transplantation in a child. *Surg Endosc* 2018; **32**: 513 [PMID: 28717867 DOI: 10.1007/s00464-017-5692-9]

74   **Roll GR**, Parekh JR, Parker WF, Siegler M, Pomfret EA, Ascher NL, Roberts JP. Left hepatectomy versus right hepatectomy for living donor liver transplantation: shifting the risk from the donor to the recipient. *Liver Transpl* 2013; **19**: 472-481 [PMID: 23447523 DOI: 10.1002/lt.23608]

75   **Chan SC**, Fan ST, Lo CM, Liu CL. Effect of side and size of graft on surgical outcomes of adult-to-adult live donor liver transplantation. *Liver Transpl* 2007; **13**: 91-98 [PMID: 17192891 DOI: 10.1002/lt.20987]

76   **Chan SC**, Lo CM, Chik BH, Chow LC, Fan ST. Flowmetry-based portal inflow manipulation for a small-for-size liver graft in a recipient with spontaneous splenorenal shunt. *Clin Transplant* 2010; **24**: 410-414 [PMID: 19807745 DOI: 10.1111/j.1399-0012.2009.01100.x]

77   **Chan SC**, Lo CM, Chok KS, Sharr WW, Cheung TT, Tsang SH, Chan AC, Fan ST. Modulation of graft vascular inflow guided by flowmetry and manometry in liver transplantation. *Hepatobiliary Pancreat Dis Int* 2011; **10**: 649-656 [PMID: 22146631]

78   **Au KP**, Chan SC, Chok KS, Chan AC, Wong TC, Sharr WW, Lo CM. Durability of small-for-size living donor allografts. *Liver Transpl* 2015; **21**: 1374-1382 [PMID: 26123155 DOI: 10.1002/lt.24205]

79   **Soejima Y**, Shirabe K, Taketomi A, Yoshizumi T, Uchiyama H, Ikegami T, Ninomiya M, Harada N, Ijichi H, Maehara Y. Left lobe living donor liver transplantation in adults. *Am J Transplant* 2012; **12**: 1877-1885 [PMID: 22429497 DOI: 10.1111/j.1600-6143.2012.04022.x]

80   **Giulianotti PC**, Coratti A, Angelini M, Sbrana F, Cecconi S, Balestracci T, Caravaglios G. Robotics in general surgery: personal experience in a large community hospital. *Arch Surg* 2003; **138**: 777-784 [PMID: 12860761 DOI: 10.1001/archsurg.138.7.777]

81   **Chen PD**, Wu CY, Wu YM. Use of robotics in liver donor right hepatectomy. *Hepatobiliary Surg Nutr* 2017; **6**: 292-296 [PMID: 29152475 DOI: 10.21037/hbsn.2017.01.17]

82   **Wakabayashi G**, Nitta H, Takahara T, Shimazu M, Kitajima M, Sasaki A. Standardization of basic skills for laparoscopic liver surgery towards laparoscopic donor hepatectomy. *J Hepatobiliary Pancreat Surg* 2009; **16**: 439-444 [PMID: 19458893 DOI: 10.1007/s00534-009-0122-6]

83   **Belghiti J**, Guevara OA, Noun R, Saldinger PF, Kianmanesh R. Liver hanging maneuver: a safe approach to right hepatectomy without liver mobilization. *J Am Coll Surg* 2001; **193**: 109-111 [PMID: 11442247]

84   **Kim JH**, Ryu DH, Jang LC, Choi JW. Lateral approach liver hanging maneuver in laparoscopic anatomical liver resections. *Surg Endosc* 2016; **30**: 3611-3617 [PMID: 26541742 DOI: 10.1007/s00464-015-4663-2]

85   **Pringle JH**. V. Notes on the Arrest of Hepatic Hemorrhage Due to

Trauma. *Ann Surg* 1908; **48**: 541-549 [PMID: 17862242]

86   **Imamura H**, Takayama T, Sugawara Y, Kokudo N, Aoki T, Kaneko J, Matsuyama Y, Sano K, Maema A, Makuuchi M. Pringle's manoeuvre in living donors. *Lancet* 2002; **360**: 2049-2050 [PMID: 12504404]

87   **Miller CM**, Masetti M, Cautero N, DiBenedetto F, Lauro A, Romano A, Quintini C, Siniscalchi A, Begliomini B, Pinna AD. Intermittent inflow occlusion in living liver donors: impact on safety and remnant function. *Liver Transpl* 2004; **10**: 244-247 [PMID: 14762862 DOI: 10.1002/lt.20071]

88   **Park JB**, Joh JW, Kim SJ, Kwon CH, Chun JM, Kim JM, Moon JI, Lee SK. Effect of intermittent hepatic inflow occlusion with the Pringle maneuver during donor hepatectomy in adult living donor liver transplantation with right hemiliver grafts: a prospective, randomized controlled study. *Liver Transpl* 2012; **18**: 129-137 [PMID: 21837746 DOI: 10.1002/lt.22409]

89   **Ishizawa T**, Saiura A, Kokudo N. Clinical application of indocyanine green-fluorescence imaging during hepatectomy. *Hepatobiliary Surg Nutr* 2016; **5**: 322-328 [PMID: 27500144 DOI: 10.21037/hbsn.2015.10.01]

90   **Mizuno S**, Isaji S. Indocyanine green (ICG) fluorescence imaging-guided cholangiography for donor hepatectomy in living donor liver transplantation. *Am J Transplant* 2010; **10**: 2725-2726 [PMID: 21062417 DOI: 10.1111/j.1600-6143.2010.03288.x]

91   A prospective analysis of 1518 laparoscopic cholecystectomies. The Southern Surgeons Club. *N Engl J Med* 1991; **324**: 1073-1078 [PMID: 1826143 DOI: 10.1056/NEJM199104183241601]

92   **Yu YD**, Kim KH, Jung DH, Lee SG, Kim YG, Hwang GS. Laparoscopic live donor left lateral sectionectomy is safe and feasible for pediatric living donor liver transplantation. *Hepatogastroenterology* 2012; **59**: 2445-2449 [PMID: 22466713 DOI: 10.5754/hge12134]

Basic Study

Intra-individual comparison of therapeutic responses to vascular disrupting agent CA4P between rodent primary and secondary liver cancers

Ye-Wei Liu, Frederik De Keyzer, Yuan-Bo Feng, Feng Chen, Shao-Li Song, Johan Swinnen, Guy Bormans, Raymond Oyen, Gang Huang, Yi-Cheng Ni

Ye-Wei Liu, Gang Huang, Shanghai Key Laboratory for Molecular Imaging, Shanghai University of Medicine and Health Sciences, Shanghai 201318, China

Ye-Wei Liu, Frederik De Keyzer, Yuan-Bo Feng, Feng Chen, Johan Swinnen, Guy Bormans, Raymond Oyen, Yi-Cheng Ni, Biomedical Group, Campus Gasthuisberg, KU Leuven, Leuven 3000, Belgium

Ye-Wei Liu, Shao-Li Song, Gang Huang, Institute of Clinical Nuclear Medicine, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China

Ye-Wei Liu, Gang Huang, Institute of Health Sciences, Shanghai Jiao Tong University School of Medicine (SJTUSM) & Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences (CAS), Shanghai 200025, China

ORCID number: Ye-Wei Liu (0000-0002-4360-0132); Frederik De Keyzer (0000-0002-1855-3274); Yuan-Bo Feng (0000-0002-4235-3491); Feng Chen (0000-0001-8490-0054); Shao-Li Song (0000-0003-2544-7522); Johan Swinnen (0000-0002-7720-5077); Guy Bormans (0000-0002-0335-7190); Raymond Oyen (0000-0003-2256-5910); Gang Huang (0000-0003-1939-3978); Yi-Cheng Ni (0000-0002-8553-7565).

Author contributions: Ni YC, Liu YW, Huang G, Swinnen J, Bormans G and Oyen R designed the research; Liu YW, Feng YB, Chen F and Ni YC performed the research; Liu YW, De Keyzer F and Chen F analyzed the data; Liu YW, Ni YC and Song SL prepared the manuscript; Ni YC, Swinnen J, Bormans G and Oyen R reviewed the manuscript; all authors approved the final version for publication.

Institutional review board statement: This study was reviewed and approved by the ethics committee of KU Leuven.

Institutional animal care and use committee statement: This animal experiment was performed in compliance with European and national regulations after approval by KU Leuven

Correspondence to: Yi-Cheng Ni, MD, PhD, Full Professor, Theragnostic Laboratory, Department of Imaging and Pathology, Biomedical Group, KU Leuven, Herestraat 49, Leuven 3000, Belgium. yicheng.ni@kuleuven.be
Telephone: +32-16-330165
Fax: +32-16-343765

Abstract

AIM
To compare therapeutic responses of a vascular-

disrupting-agent, combretastatin-A4-phosphate (CA4P), among hepatocellular carcinomas (HCCs) and implanted rhabdomyosarcoma (R1) in the same rats by magnetic-resonance-imaging (MRI), microangiography and histopathology.

METHODS
Thirty-six HCCs were created by diethylnitrosamine gavage in 14 rats that were also intrahepatically implanted with one R1 per rat as monitored by T2-/T1-weighted images (T2WI/T1WI) on a 3.0T clinical MRI-scanner. Vascular response and tumoral necrosis were detected by dynamic contrast-enhanced (DCE-) and CE-MRI before, 1 h after and 12 h after CA4P iv at 10 mg/kg (treatment group *n* = 7) or phosphate-buffered saline at 1.0 mL/kg (control group *n* = 7). Tumor blood supply was calculated by a semiquantitative DCE parameter of area under the time signal intensity curve (AUC30). *In vivo* MRI findings were verified by postmortem techniques.

RESULTS
On CE-T1WIs, unlike the negative response in all tumors of control animals, in treatment group CA4P caused rapid extensive vascular shutdown in all R1-tumors, but mildly or spottily in HCCs at 1 h. Consequently, tumor necrosis occurred massively in R1-tumors but patchily in HCCs at 12 h. AUC30 revealed vascular closure (66%) in R1-tumors at 1 h (*P* < 0.05), followed by further perfusion decrease at 12 h (*P* < 0.01), while less significant vascular clogging occurred in HCCs. Histomorphologically, CA4P induced more extensive necrosis in R1-tumors (92.6%) than in HCCs (50.2%) (*P* < 0.01); tumor vascularity heterogeneously scored +~+++ in HCCs but homogeneously scored ++ in R1-tumors.

CONCLUSION
This study suggests superior performance of CA4P in metastatic over primary liver cancers, which could guide future clinical applications of vascular-disrupting-agents.

Key words: Hepatocellular carcinoma; Combretastatin A4 phosphate; Rhabdomyosarcoma; Vascular-disrupting agent; Magnetic resonance imaging; Rats

Core tip: Complex animal models combining primary and secondary liver malignancies proved feasible in rats. The therapeutic efficacy of the leading vascular disrupting agent combretastatin-A4-phosphate (CA4P) could be intra-individually compared between primary and secondary liver malignancies in the same cirrhotic rats. Clinical 3.0T magnetic resonance imaging allowed real-time monitoring of *in vivo* therapeutic responses within 12 h, and *ex vivo* microangiography and histopathology could validate the CA4P-induced tumoricidal effects. The therapeutic responses appeared superior with secondary liver tumors over that with primary hepatocellular carcinomas, which are of translational significance for planning future clinical trials of CA4P in cancer patients.

INTRODUCTION

As a first vascular disrupting agent (VDA), combretastatin-A4-phosphate (CA4P) targets the cytoskeletal tubulin of abnormal tumor endothelial cells, leading to a rapid but often reversible vascular occlusion[1-3]. Theoretically, this may cause ischemic tumor necrosis by depriving malignant cells from the blood supply[1-3]. Clinically, CA4P has been undergoing phase II/III trials in the setting of ovarian, thyroid and lung cancers alone or in combination with other chemotherapeutic agents[4-6], and a good safety profile has also been shown in the first phase I clinical trial among a Chinese patient population[7]. In the majority of transplanted tumor models, CA4P consistently induced massive central tumor necrosis, leaving only a few layers of peripheral viable tumor cells culpable for the incomplete treatment and cancer relapse[8,9], which is also attributed to the unsatisfactory clinical outcomes[3]. To tackle this bottleneck problem with all VDAs, a plausible solution has been proposed[10].

On the other hand, diverse and paradoxical tumor responses to CA4P have been recently noticed in a few preclinical studies based on a carcinogen-induced primary liver cancer model[11,12]. By gavage administration of diethylnitrosamine (DENA) in rodents, multifocal hepatomas of a full spectrum of tumor vascularity and cellular differentiation superimposed on various degrees of liver cirrhosis could be generated[11-14]. Compared with the ectopically and orthotopically transplanted tumors, this primary HCC model is considered to be more clinically relevant for evaluating therapeutic drugs because of the heterogeneity in tumoral microenvironment similar to that of humans[13,14], if an imaging platform can be available to accurately trace individual tumors[14,15]. In this model, CA4P simultaneously caused not only tumor necrosis but also reginal parenchymal necrosis in the cirrhotic liver[11,12].

Tumor susceptibility to VDA therapy could be largely influenced by vascular features such as vessel density, diameter, reginal instabilities in blood flow, vascular permeability and interstitial fluid pressure[16,17]. Lines of evidence have shown that, rather than larger tumor vessels, smaller or thinner ones are more susceptible

to completely shut down in response to VDAs[11,12,17]. Apart from the intrinsic properties of tumor vasculature, different tumor implantation sites and their dissimilar host-organ blood supplies may attribute to such variable efficacies of CA4P therapy as well[18,19]. Take the ectopically implanted rhabdomyosarcoma (R1) as an example; intra-individual comparisons demonstrated that hepatic R1-tumors in the intact liver responded to CA4P much better than their subcutaneous and pancreatic counterparts did[18,19]. However, issues still remain unknown as to whether R1-tumors would grow in the cirrhotic liver and whether R1-tumors growing in the cirrhotic liver are also good responders to CA4P, as they presented in the normal liver[9,10,18-21].

So far, experimental analyses of CA4P have yielded all superior results in implanted liver tumors from animals with healthy liver[9,10,18-21] and all inferior results on primary HCCs from rats with liver cirrhosis[11]. Therefore, in order to assess this potential micro-environmental impact, it would be interesting to experimentally compare the therapeutic outcomes of CA4P between primary HCCs and secondary liver tumors in the same subjects with cirrhotic livers, though such a scenario is rarely seen in clinic[22]. Accordingly, in this study we employed a DENA-induced HCC model in Wistar albino Glaxo/Rijswijk (WAG/Rij) rats that received intrahepatic transplantation of a R1-tumor to intra-individually compare the responses of different tumors to CA4P administration under the same micro-environment of liver cirrhosis. Clinical 3.0T magnetic resonance imaging (MRI) was applied for *in vivo* real-time therapeutic monitoring within 12 h, while *ex vivo* microangiography and histopathology were performed to validate the CA4P-induced outcomes.

MATERIALS AND METHODS

Animals and reagents
Male WAG/Rij rats, which are syngeneic for the cell-line of rhabdomyosarcoma (R1), weighing 300-350 g were purchased from Charles River Breeding Laboratories, Inc. (St. Aubain les Elbeuf, France). DENA (N0258) was purchased from Sigma-Aldrich (St. Louis, MO, United States). CA4P (C643025) was procured from Toronto Research Chemical Inc. (Toronto, Canada). MRI contrast agent Dotarem® (Gd-DOTA, Gadoterate meglumine; Guerbet, Villepinte, France), barium sulfate suspension (Micropaque®; Guerbet) and gas anesthetic isoflurane (Forane®; Baxter Healthcare, Deerfield, IL, United States) were also commercially obtained.

Experimental design
All animal experiments were approved by the ethics committee of KU Leuven University and performed in compliance with European and national regulations. *In vivo* procedures including gavage feeding, drug injection and MRI were carried out under gas anesthesia with 2% isoflurane (Harvard Apparatus, Holliston, MA, United States), while the laparotomy of intrahepatic R1-tumor implantation was carried out under general anesthesia with intraperitoneal injection of pentobarbital (Nembutal; Sanofi Sante Animale, Brussels, Belgium) at 50 mg/kg.

As illustrated in Figure 1, multifocal primary hepatomas superimposed on liver cirrhosis were induced in rats by 14-wk oral gavage of DENA at 5 mg/kg/d using a 16 cm-long flexible plastic esophageal gastric tube (Fuchigami Kikai, Kyoto, Japan)[13]. Tumor growth was monitored weekly by T2WI and T1WI from the 9th week until the largest liver tumor diameter reached more than 5 mm. A R1-tumor tissue block of 1 mm³ was implanted into the lower part of median liver lobe by laparotomy. Tumor growth was monitored weekly by MRI until R1 reached more than 5 mm in diameter. Next, all recruited tumor-carrying rats were randomly divided into sham group and CA4P-treated group. Seven rats in the CA4P group were intravenously injected with CA4P at 10 mg/kg, while the other 7 rats in the sham group intravenously received phosphate buffered saline (PBS) at 1 mL/kg. Multiparametric MRI was performed 4 h before and 1 h and 12 h after the CA4P/PBS treatment. Rats were sacrificed immediately after the last time point of MRI scanning for postmortem microangiography and histopathology.

In vivo MRI
A clinical 3.0T scanner (MAGNETOM Prisma; Siemens, Erlangen, Germany) and a human wrist coil (Hand/Wrist 16, A 3T Tim coil; Siemens) were used for imaging acquisition. To monitor tumor growth, T2-weighted (repetition time, 4000 ms; echo time, 70 ms; flip angle, 150°; field of view, 75 × 56 mm²; matrix, 256 × 192; acquisition time, 3.4 min) and T1-weighted (repetition time, 626 ms; echo time, 15 ms; flip angle, 160°; field of view, 75 × 56 mm²; matrix, 256 × 192; acquisition time, 3.8 min) turbo spin echo images (T2WI, T1WI) were performed weekly. Sixteen axial images with a slice thickness of 2.2 mm and a gap of 0.4 mm were acquired.

To evaluate tumor responses to CA4P treatment, T2WI, T1WI, dynamic contrast-enhanced (DCE) and consecutive CE-T1WIs were performed. DCE was conducted by a T1-weighted gradient echo (GE) sequence (repetition time, 7 ms; echo time, 2.45 ms; flip angle, 15°; field of view, 61 × 89 mm²; and matrix, 132 × 192) with 60 measurements in total acquisition time of 7.3 min. During DCE, an intravenous bolus of 0.02 mmol/kg Gd-DOTA was injected after the first 17 precontrast baseline measurements that were continued with 43 postcontrast measurements. Then, an intravenous bolus of 0.2 mmol/kg Gd-DOTA was injected, followed by consecutive CE-T1WI measurements.

MRI analyses
Images were analyzed with an off-line Siemens workstation and MeVisLab (version 2.6.2; MeVis Medical Solutions AG, Bremen, Germany). All the following measurements were conducted by three authors with
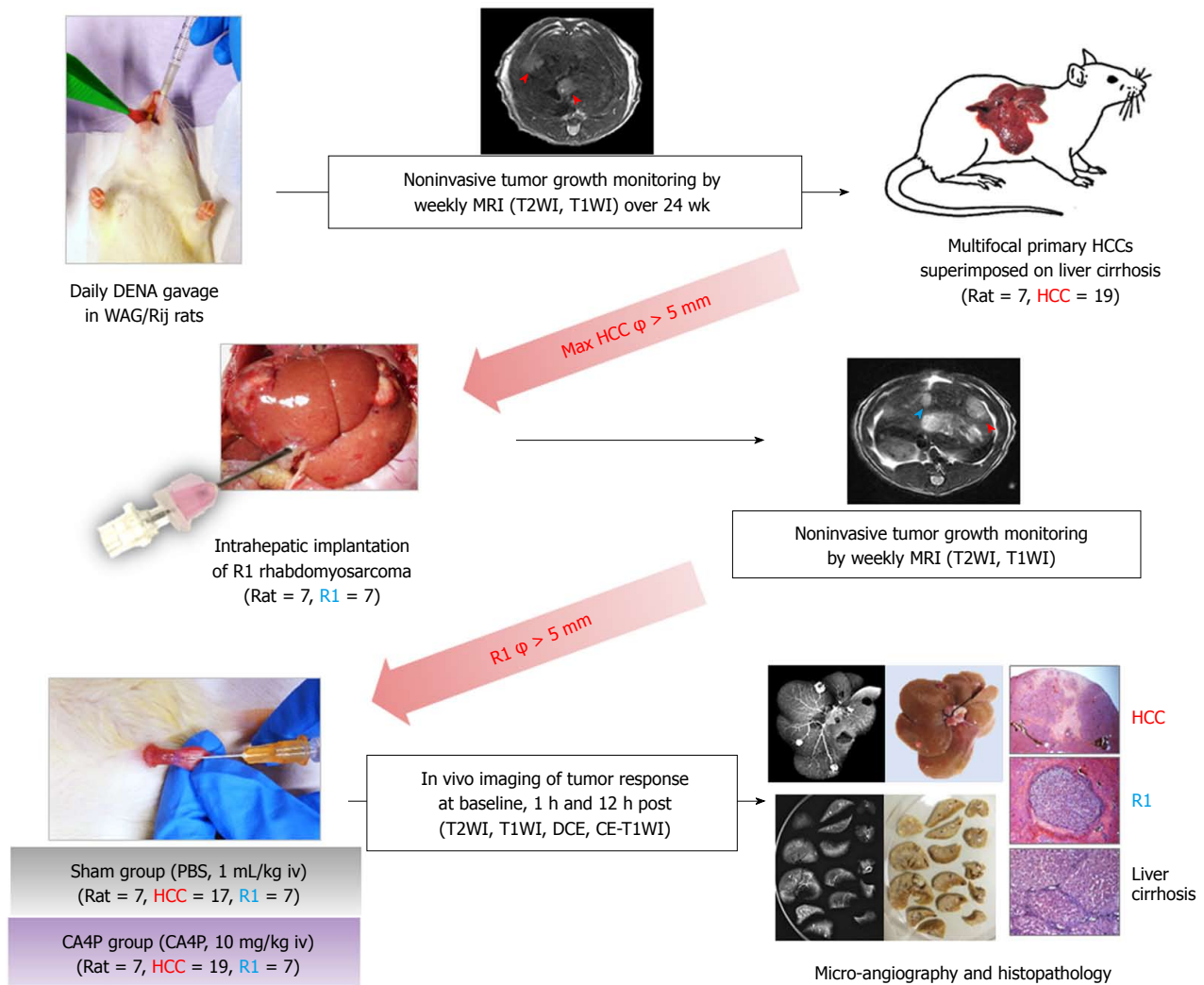
Noninvasive tumor growth monitoring by weekly MRI (T2WI, T1WI) over 24 wk

Daily DENA gavage in WAG/Rij rats

Multifocal primary HCCs superimposed on liver cirrhosis (Rat = 7, HCC = 19)

Max HCC φ > 5 mm

Intrahepatic implantation of R1 rhabdomyosarcoma (Rat = 7, R1 = 7)

Noninvasive tumor growth monitoring by weekly MRI (T2WI, T1WI)

R1 φ > 5 mm

Sham group (PBS, 1 mL/kg iv) (Rat = 7, HCC = 17, R1 = 7)

CA4P group (CA4P, 10 mg/kg iv) (Rat = 7, HCC = 19, R1 = 7)

In vivo imaging of tumor response at baseline, 1 h and 12 h post (T2WI, T1WI, DCE, CE-T1WI)

HCC

R1

Liver cirrhosis

Micro-angiography and histopathology

Figure 1 Flow chart of experimental protocol. φ: Diameter; CA4P: Combretastatin-A4-phosphate; CE: Contrast-enhanced; DCE: Dynamic contrast-enhanced; DENA: Diethylnitrosamine; HCC: Hepatocellular carcinoma; iv: Intravenous(ly); MRI: Magnetic resonance imaging; PBS: Phosphate-buffered saline; R1: R1 rhabdomyosarcoma; T1WI: T1-weighted imaging; T2WI: T2-weighted imaging; WAG/Rij rat: Wistar Albino Glaxo/Rijswijk rat.

consensus.

Tumor diameter: On T2WI, the tumor was manually contoured on the lesion-containing slices and tumor volume was automatically generated by the software, on which the tumor diameter was obtained.

Semiquantitative analysis of T1-weighted DCE: For DCE analysis, namely AUC30 calculation, the operator-defined region of interest (ROI) of tumor was freehand delineated on all tumor-containing slices. ROI of abdominal aorta was delineated from four consecutive slices for defining arterial input function. ROI of the liver was delineated on four representative slices each from median, left, right and caudate lobes. All ROIs were automatically copied to all measurements. Because of a low gadolinium dose, a linear relation between the amount of contrast agent in the tissue and the resultant difference in relaxation time could been assumed[23]. As a robust semiquantitative DCE parameter against movements, area under the time signal intensity curve

(AUC30) was calculated to reflect tumor blood flow[24].

Digital microangiography

After the last MRI scan, rats were anesthetized by an intraperitoneal injection of pentobarbital at 50 mg/kg. A laparotomy was performed with abdominal aorta cannulated, through which barium suspension was injected before the entire tumor-bearing liver was excised. Postmortem hepatic arteriography was conducted by a digital mammography unit (Em-brace; Agfa-Gevaert, Mortsel, Belgium) at 26 kV and 32 mAs. Then, the livers were fixed and sliced into 3-mm sections in the axial plane corresponding to the MR images, before being radiographed at 26 kV and 18 mAs for qualitative validation of tumor vascularity.

Histopathology

After microangiography, the tissue sections were paraffin-imbedded, sliced and stained with hematoxylin and eosin (HE) for microscopic analyses using an Axiovert 200M microscope equipped with an AxioCam MR monochrome

digital camera (Carl Zeiss Inc, Gottingen, Germany) and AxioVision 4.8 software.

Calculation of CA4P-induced intratumoral necrosis: Microscopic images of H&E-stained tumor slices at a magnification of 12.5 were used to estimate the percentage of intratumoral necrosis by using ImageJ software[25]. To obtain the 'necrotic ratio on each section', ROIs around the entire tumor and the necrotic tumor were manually delineated, respectively. The sectional tumor area of each 3-mm tumor section was measured and represented as the axial side of this tumor block with the largest diameter. Tumor necrosis was estimated independently by two pathologists, and calculated with the equation: Intratumoral necrosis ratio (%) = $\sum$ [Necrotic ratio on each section (%) × section area (mm^2)] × section thickness (mm) / [4/3π r^3] (mm^3).

Grading of HCC differentiation: In view of the high analogy to histopathological features in human liver cancer, rat HCCs were diagnosed according to the classical histomorphologic features of malignant hepatocytic tumors, often well vascularized, with wide trabeculae (> 3 cell layers), noticeable acinar pattern, small cell changes, cytologic atypia, prominent nucleoli, mitotic activity, vascular invasion, absence of Kupffer cells, lack of portal triad, and loss of the reticulin network[26]. The differentiation of rat HCCs was further graded using a modified 4-scale Edmondson and Steiner system[26] as the standard criteria, as follows: grade I, highly differentiated, consisting of tumor cells of moderate size arranged in thin trabeculae; grade II, larger cells with active nuclear mitosis and possible pseudoglandular structures often with steatosis; grade III, larger nuclei and more hyperchromatic or increased mitotic figures, granular and acidophilic cytoplasm, often with giant tumor cells; and grade IV, much less differentiated tumor cells with hyperchromatic nuclei and loss of trabecular pattern often with angioinvasion[26].

Grading of tumor vascularity: To characterize variable degrees of tumoral vascularity, a semiquantitative vascular scoring system was adopted to classify HCCs as follows: +, similar vascular density to the liver parenchyma; ++, dense vasculature without vascular lakes; +++, denser vasculature with variously sized vascular lakes; and ++++, full of enlarged vascular lakes[11,12].

Statistical analysis

Numerical data were expressed as the mean ± standard error of the mean (SEM) and a significant difference was concluded for *P* < 0.05. *In vivo* imaging biomarker AUC30 at different time points and postmortem tumoral necrosis were compared between HCC and liver R1 by unpaired two-tailed *t*-test using GraphPad Prism (version 7.02; GraphPad Software Inc., La Jolla, CA, United States).

RESULTS

General aspects

In general, all rats tolerated the experimental procedures well, including gas anesthesia, DENA gavage, MRI scanning, laparotomy of intrahepatic tumor implantation, contrast administration and intravenous CA4P/PBS treatment. In total, 19 primary HCCs and 7 hepatic R1 allografts were successfully established in the 7 rats of the CA4P group (Table 1), while 17 primary HCCs and 7 R1-tumors were generated in the 7 rats of the sham group. The rats were sacrificed 12 h after CA4P/PBS treatment when CA4P-induced tumor necrosis was most evident.

Uniform versus variable vascularity between hepatic R1 allografts and primary HCCs

Similar to the previous findings in Sprague Dawley rats[27], various tumoral vascularity and cellular differentiation of primary HCCs were discovered in the WAG/Rij rats (Table 1). Yet, vascularity of HCCs mainly appeared as grade +~++, probably due to a lower-dosed DENA gavage (5 mg/kg/d *vs* 10 mg/kg/d) but a prolonged administration period (150 d *vs* 90 d) in addition to the different species. In contrast, vascularity of intrahepatic R1 allografts was uniformly identified as grade ++ (Table 1), similar to that of other tumor studies on different animal strains[9,10,18-21].

Tumoricidal effects in metastatic R1-tumors versus heterogeneous responses in primary HCCs

In vivo real-time responses of primary HCCs and R1 allografts were visualized by multiparametric MRI prior to, and 1 and 12 h posttreatment. At baseline for the CA4P group and all time points for the sham group, hepatic R1 nodules appeared highly hyperintense on T2WIs (Figures 2A1, 3A1, 2D1), iso- to slightly hyperintense on precontrast T1WIs (Figures 2A2, 3A2, 2D2) and homogeneously hyper-enhanced on CE-T1WIs (Figures 2A3, 3A3, 2D3) compared with the liver parenchyma. Additionally, spontaneous necrosis existing in hepatic R1 of Rat 3 was indicated by the unenhanced area on CE-T1WI at baseline (Figure 3A3). Intra-individually, their paired primary HCCs on the same imaging slice appeared moderately hyperintense on T2WIs (Figures 2A1, 3A1, 2D1') as well as on precontrast T1WIs (Figures 2A2, 3A2, 2D2'), and hyper-enhanced on CE-T1WIs (Figures 2A3, 3A3, 2D3').

At 1 h after CA4P treatment, despite nearly unchanged intensities of hepatic R1 allografts on T2WIs (Figures 2A1', 3A1') and T1WIs (Figures 2A2', 3A2'), signals on CE-T1WIs were distinctly altered by an unenhanced central region surrounded by a positively enhanced periphery (Figures 2A3', 3A3'), indicative of ongoing extensive vascular shutdown. Nevertheless, the contrast of the primary HCC counterparts was slightly enhanced in a heterogeneous pattern (Figures 2A3', 3A3').

At 12 h, massive central necrosis occurred in

Table 1 Intra-individual comparison of induced tumor necrosis (%) between primary hepatocellular carcinomas and intrahepatically implanted R1 rhabdomyosarcomas in combretastatin-A4-phosphate-treated group

Rat	Primary HCC					Implanted hepatic R1			
	Tumor code	CA4P-induced necrosis, %	Tumor diameter in mm	Tumor vascularity[1]	Tumor differentiation[2]	Tumor code	CA4P-induced necrosis, %	Tumor diameter in mm	Tumor vascularity[1]
1	HCC_1	21.8	9.7	++	II	R1_1	72.3	12.1	++
	HCC_2	16.4	6.5	++	III-IV				
	HCC_3	0	10.9	++	III				
2	HCC_4	43.1	6.4	+	III	R1_2	84.5	12.6	++
	HCC_5	23.3	8.5	++	III				
3	HCC_6	92.3	8.1	+	I-II	R1_3	99.2	10	++
	HCC_7	96.5	6.2	+	II				
	HCC_8	19.8	10	+	I				
	HCC_9	98.9	10	+	II				
4	HCC_10	99.2	14.3	+	I-II	R1_4	96.8	9.8	++
5	HCC_11	27.6	18.3	+	III	R1_5	99.4	8.3	++
	HCC_12	4.9	7.8	++	II-III				
	HCC_13	62.7	13	+	I-II				
6	HCC_14	47.6	14.2	+, +++[3]	I, III[4]	R1_6	97.7	9	++
	HCC_15	46.4	14.2	+, +++[3]	I, III[4]				
7	HCC_16	76.1	12.5	+	II-III	R1_7	98.3	6.2	++
	HCC_17	552.6	11.9	+	III				
	HCC_18	33.4	10.4	+	III				
	HCC_19	91.2	9	+	I-II				
Mean ± SD		50.2 ± 1.8	10.6 ± 0.2	/	/		92.6 ± 1.5	9.7 ± 0.3	/

[1]A vascular scoring system for rat liver tumor: vascular density similar to that of liver parenchyma (+), denser vasculature without vascular lakes (++), denser vasculature with small-sized vascular lakes (+++), and full of large vascular lakes (++++); [2]A 4-scale grading system for HCC differentiation in rats: Well (I), moderately (II), poorly (III) and un-(IV) differentiated HCC lesions; [3]Tumor vascularity was graded as + in the necrotic tumor, and +++ in the residual viable part; [4]HCC differentiation was scored by I in the necrotic tumor, and III in the residual viable part. HCC: Hepatocellular carcinoma; SD: Standard deviation.

all the hepatic R1 tumors, as reflected by extreme hyperintensity on T2WIs (Figure 2A1"), isointensity on T1WIs (Figures 2A2", 3A2") and an unenhanced core surrounded by a hyperenhanced rim on CE-T1WIs (Figures 2A3", 3A3"). Meanwhile, by comparison, patchy necrosis was heterogeneously induced in primary HCCs, shown as generally increased hyperintensity on T2WIs (Figures 2A1", 3A1"), mingled hyper- and isointensities on T1WIs (Figures 2A2'', 3A2'') and regional unenhancement scattering in extremely hyperenhanced lesions on CE-T1WIs (Figures 2A3", 3A3").

These *in vivo* imaging findings were eventually confirmed by postmortem microangiography and histopathology. At 12 h, complete absence of tumor vessels was particularly identified in the center of hepatic R1 (Figures 2B and 3B), whereas in primary HCCs, generally denser vasculature was mixed with patchy avascular areas (Figures 2B and 3B). From HE-stained slices, massive hemorrhagic necrosis and focal necrosis were indicated in hepatic R1 and in primary HCCs, respectively (Figures 2C and 3C).

Meanwhile, in the sham group (Figure 2D), *in vivo* MRI did not show any obvious difference 4 h before, and 1 and 12 h after PBS injection. From postmortems, no vascular changes were microangiographically identified, and no acute tumoral necrosis was histopathologically discovered.

Quantitative changes of tumor blood supply in correlation to CA4P-induced necrosis
Real-time changes of tumor blood supply after CA4P administration were monitored *in vivo*.

DCE-MRI: As reflected by AUC30 (Figure 4A), blood flow in hepatic R1-tumors dropped by 66% at 1 h due to vascular shut-down, followed by a further reduction of 7.3% at 12 h as a result of massive tumoral necrosis (Figure 4B). Nevertheless, in primary HCCs, only 11% tumor blood flow was reduced at 1 h because of vascular clogging, followed by a slight resumption of tumor perfusion at 12 h (Figure 4B), which was a heterogeneous combination of partial tumoral necrosis and reopening of large intra-tumoral vessels in residual tumor. As validated by histopathological analysis, tumoral necrosis in liver R1 allografts (92.6%) was more extensive than that in primary HCCs (50.2%) at 12 h after CA4P treatment (Figure 4C, Table 1).

Taken together, these intra-individual comparisons demonstrated that in general CA4P caused more extensive tumor vascular destruction and consequent tumoral necrosis in intrahepatically implanted R1-tumors than in the primary HCC lesions, both under the same cirrhotic liver background.

DISCUSSION

To the best of our knowledge, this is the first study where (1) a rat tumor model combining primary HCCs and an implanted R1-tumor in the same cirrhotic liver has thus been established and (2) the therapeutic efficacies of a VDA CA4P on distinct tumor types have been intra-individually compared. This, together with
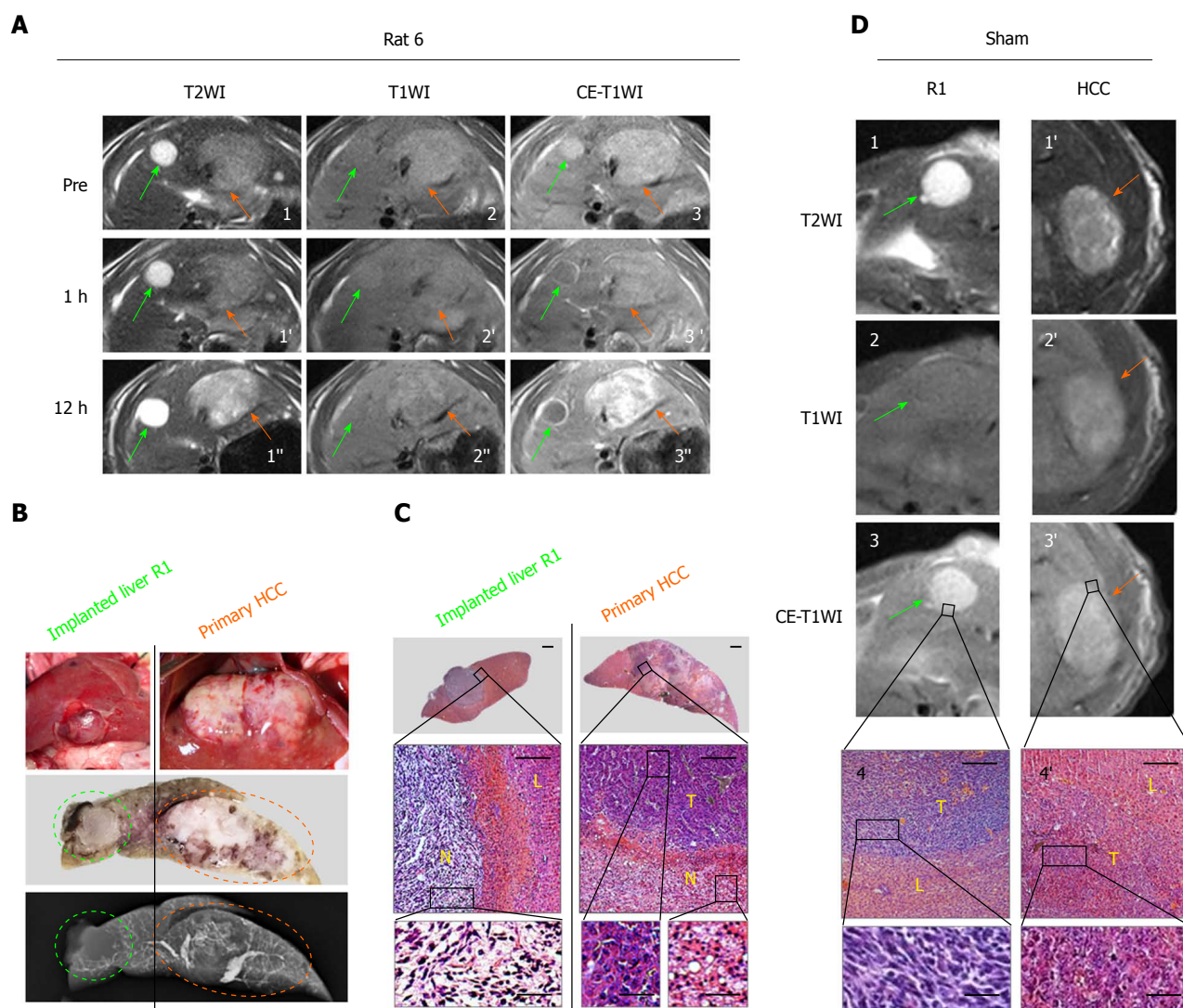
Figure 2 Intra-individual comparison of therapeutic responses to combretastatin-A4-phosphate between a primary hepatocellular carcinoma and a hepatic R1 allograft located in different liver lobes. A: T2WIs (1-1''), T1WIs (2-2') and CE-T1WIs (3-3'') of an implanted R1-tumor (green arrows) and a primary HCC (orange arrows) located in the median and left liver lobes, respectively, at baseline and 1 h and 12 h after CA4P therapy; B: Corresponding photomacrographs of median and left liver lobes (top panels), photomacrograph of liver blocks (middle panel) in 2-mm thickness corresponding to the transversal MRI, and microangiogram (bottom panel) of tumor-bearing liver blocks, revealing one R1-tumor (green circle) and one primary HCC (orange circle); C: Corresponding photomicrographs of R1-tumor (left column) and primary HCC (right column) in the median and left lobes, respectively. (HE staining; upper panels, × 12.5 original magnification, scale bar = 800 μm; lower panels, × 100 original magnification, scale bar = 100 μm, × 400 original magnification, scale bar = 25 μm); D: Sham control: T2WIs (1, 1'), T1WIs (2, 2') and CE-T1WIs (3, 3') of R1-tumor (green arrows) and primary HCC (orange arrows) located in the median and left liver lobes, respectively, at 12 h post PBS treatment, and corresponding photomicrographs (4, 4'; HE staining × 100 original magnification, scale bar = 100 μm, × 400 original magnification, scale bar = 25 μm). HCC: Hepatocellular carcinoma; L: Liver; N: Tumoral necrosis; PBS: Phosphate-buffered saline; T: Viable tumor.

the applied MRI-microangiography-histology platform, could be regarded as methodological advances for conducting more efficient theragnostic investigations on spontaneous *vs* metastatic liver malignancies.

This unique rat model of primary and secondary liver tumors induced by a carcinogen and surgery was employed not only to closely mimic the synchronous primary and metastatic liver malignancies seen in clinical patients, though of rarity[22], but also to better compare such complex liver cancers, especially in terms of different tumor differentiation, angiogenesis and vasculature, towards the same therapeutics of CA4P.

Based on the fact that the target of CA4P is tumoral vasculature rather than cancer cells, transplanted

R1 rhabdomyosarcoma is a suitable model of secondary hepatic tumor because of the similar tumor neovascularization process and the existing vasculature pattern to those intrahepatic metastases[15]. Transplanted R1-tumor is a type of homogeneous, hypervascularized, solid tumor, with abundant microvessels[14]. Although in patients intrahepatic metastases occurring *via* the hematogenous route, they always end up with the same consequence of tumor neovascularization. Therefore, the derived results are representative of that in other metastatic liver tumors from different original sites.

Unlike ectopically and orthotopically transplanted tumor models that yield reproducible outcomes, experimental models of primary liver malignancies tend
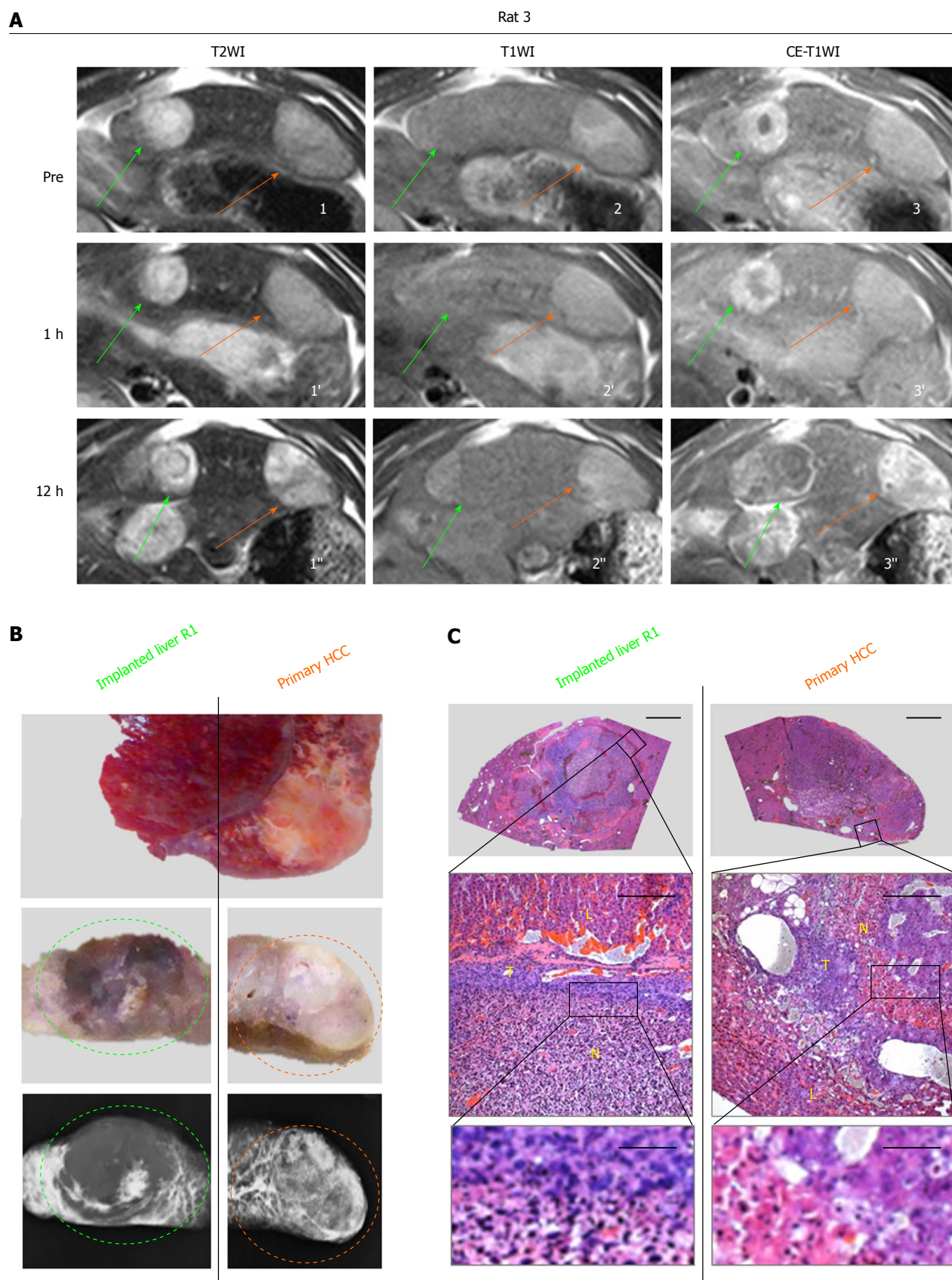
Figure 3 Intra-individual comparison of therapeutic responses to combretastatin-A4-phosphate between a primary hepatocellular carcinoma and a hepatic R1 allograft distributed in the same liver lobe. A: T2WIs (1-1''), T1WIs (2-2'') and CE-T1WIs (3-3'') of an implanted R1-tumor (green arrows) and a primary HCC (orange arrows) both located in the same left liver lobe at baseline and 1 h and 12 h after CA4P therapy; B: Corresponding macroscopic photographs of the left liver lobe (top panel) and liver blocks (middle panels) in 2-mm thickness corresponding to the transversal MRI, and microangiograms (bottom panels) of tumor-bearing liver block, revealing a R1-tumor (green circle) and a primary HCC (orange circle); C: Corresponding photomicrographs of R1-tumor (left column) and primary HCC (right column). (HE staining; upper panels, × 12.5 original magnification, scale bar = 800 μm; lower panels, × 100 original magnification, scale bar = 100 μm, × 400 original magnification, scale bar = 25 μm). HCC: Hepatocellular carcinoma; L: Liver; N: Tumoral necrosis; T: Viable tumor.
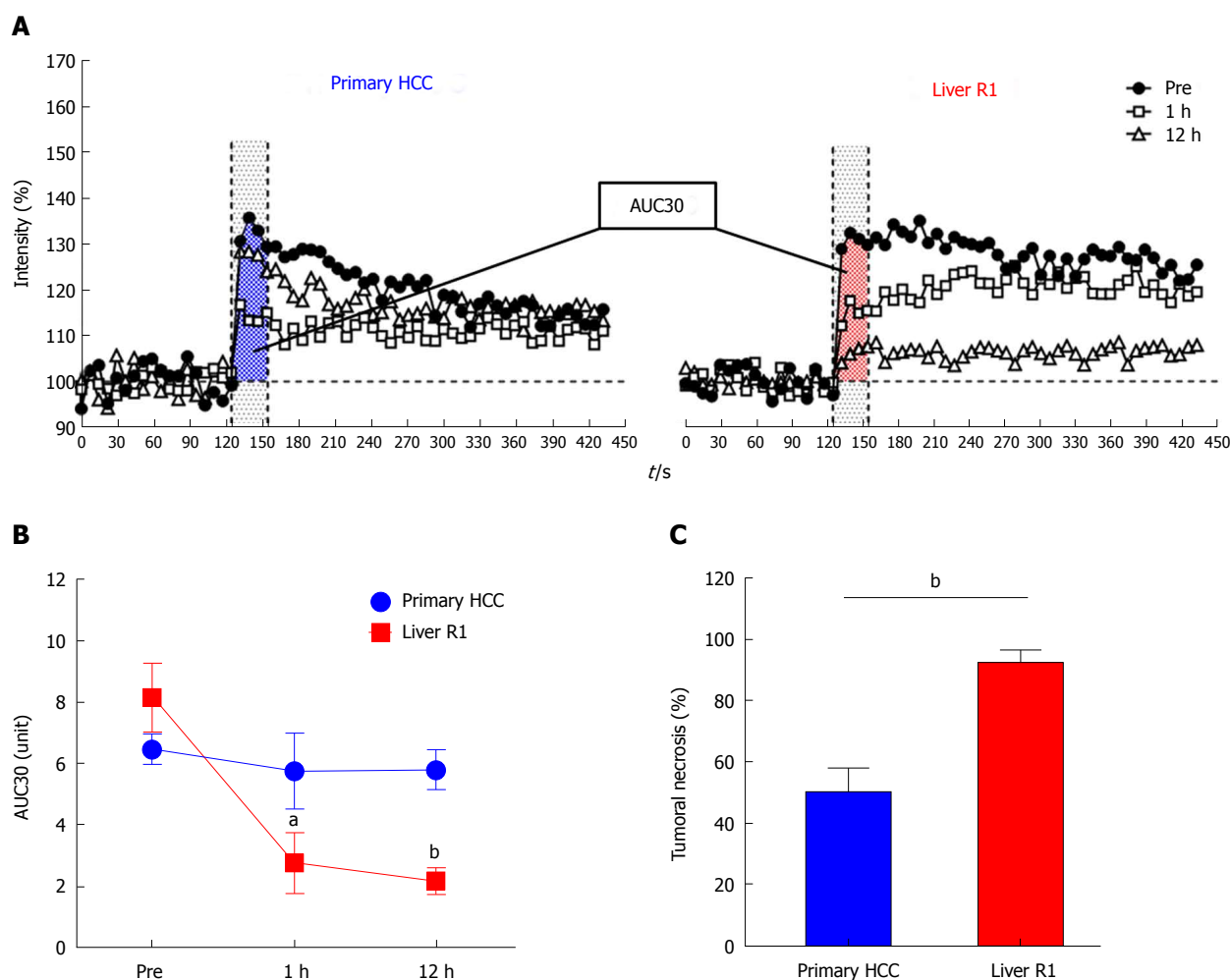
A

B

C

Figure 4 Changes of semiquantitative dynamic contrast-enhanced parameter of primary hepatocellular carcinomas and implanted liver R1-tumors and quantification of combretastatin-A4-phosphate-induced tumoral necrosis. A: Representative contrast enhancement-time curves of a primary HCC and a secondary liver R1-tumor before, and 1 h and 12 h after CA4P treatment, for calculating tumor AUC30 at different time points; B: Quantitative changes of tumor blood supply between HCCs and R1-tumors at baseline and 1 h and 12 h after CA4P treatment indicated by AUC30; C: Bar chart comparing the percentile tumoral necrosis between primary HCCs and implanted liver R1 at 12 h after CA4P therapy, which was estimated by postmortem HE staining. [a]$P < 0.05$, [b]$P < 0.01$. AUC30: Area under the time signal intensity curve; HCC: Hepatocellular carcinoma.

to be more therapeutically and histologically unpredictable owing to intra- and inter-tumoral heterogeneity[11,12]. Particularly, despite undergoing similar carcinogenesis, DENA-induced primary HCCs exhibit huge diversities in carcinoma development, neovascularization or tumor vascularity, microenvironment and cellular differentiation in addition to varied degrees of liver cirrhosis[11,12,14]. Therefore, while constructing both primary and implanted tumors could be more time-consuming and technically challenging [13], this complex liver tumor model appears more clinically relevant for mimicking miscellaneous human cancers[14,22].

In this study, distinct responses to CA4P, namely more complete tumoricidal effect on implanted R1-tumors *versus* variable outcomes in primary HCCs, simultaneously occurred in the same rats with cirrhotic livers. These findings are in alignment with the previous studies conducted in either DENA-induced primary HCC model on cirrhotic liver[11,12] or implanted R1-tumor model in normal liver[9,10,20,21]. Thus, the role of cirrhotic or normal liver background in the therapeutic impact

of CA4P could be basically excluded. It was more likely that the intrinsic vasculature of the individual tumors eventually determined various outcomes of CA4P therapy. Indeed, as a widely accepted notion, implanted liver tumors resemble more closely the secondary or metastatic liver cancer[15]. Therefore, our results strongly indicate that, in general, CA4P exerts more potent therapeutic effects on the metastatic liver tumors, rather than the primary liver tumors.

In principle, tumor angiogenesis switches on when a tumor reaches 1 mm^3 in volume, since this is the limited size of diffusion within which solid tumor cells can grow[28]. Apart from the basic type of angiogenesis, namely endothelial sprouting, there are several nonangiogenic tumor vascularization mechanisms, including vasculogenic mimicry, intussusception and vascular co-option[29,30]. Vasculogenic mimicry refers to tumor cells mimicking endothelial cells and directly participating in blood vessel formation, while intussusception and vascular co-option are both vascularization modes that essentially take advantage of the existing vasculature in the surrounding

benign tissue[29,30]. For instance, in experimental liver metastatic model produced by splenic injection of CD38 colon carcinoma cells in mice, enlarged sinusoidal lakes were discovered to be developed by fusion of the normal structure of sinusoids[31]. Since primary HCCs are generally hypervascularized tumors[32], vascularization based on remodeling of the existing blood vessels is more complicated, especially in terms of enlarged vascular lakes. These lines of evidence may explain to some extent the heterogeneous vasculature observed in our primary HCC model that developed gradually in the context of cirrhotic liver[11]. In support of this, by treating rats with DENA in a lower dose and a longer exposure period, less severe liver cirrhosis along with lower grades of tumor vascularity and HCC differentiation were identified in this study, as compared to a previous study[11].

Liver cirrhosis is considered as a precancerous condition since over 80% HCCs arise on a background of cirrhosis[26,33]. In fact, the progression of cirrhosis is accompanied by a deformation of the hepatic vasculature in regenerated lobules[34]. Consequent hepatic vascular alterations include shunting of the portal and arterial blood directly into the central vein, compromising exchange between hepatic sinusoids and the adjacent liver parenchyma, and disturbed hepato-biliary excretion[32,34]. In the context of cirrhosis, distorted neovasculature not only functioned as a unique mode of blood supply but also appeared to be responsive to CA4P treatment, leading to patchy necrosis in cirrhotic liver paranchyma[12]. Hence, vigilance should be exercised when using VDAs in patients with extensive liver cirrhosis, since acute necrosis in liver parenchyma could further impair hepatic function.

Currently, although a series of phase II/III clinical trials have aimed at evaluating the treatment of CA4P in combination with chemotherapy in ovarian cancer[4], anaplastic thyroid cancer[5] and nonsquamous non-small cell lung cancer patients[6], CA4P still literally remains an investigational medicine. The fetter that prevents CA4P from being ultimately adopted as a clinical anticancer therapy lies in tumor regrowth after monotherapy[35], despite its prompt, effective and generic responses in almost all solid tumors. Hence, combining CA4P with sequential treatments like chemotherapy, conventional radiotherapy, internal targeted radiotherapy and antiangiogenic therapy could reinvigorate these VDAs and provide better long-term outcomes. In fact, a dual-targeting pan-anticancer theragnostic approach called OncoCiDia using CA4P sequentially with a radioiodinated necrosis avid compound, ^{131}I-hypericin, has been proposed to achieve CA4P-induced necrosis-oriented internal targeted radiotherapy[10,36]. In this context, prior to setting serial VDA-centric anticancer protocols, the present synchronous multiple liver cancer model in rodents could be a stepping-stone to help predict the diverse responses that may occur in patients, and to further address more complicated clinically relevant questions[22]. For instance, to those patients with the

HCCs less responsive to CA4P, alternatives such as radiofrequency ablation, microwave ablation and high intensity focused ultrasound can be applied to massively necrotize the tumor before systemic administration of a necrosis-avid radiopharmaceutical in the OncoCiDia strategy[10,36].

In conclusion, this study suggests distinct responses to CA4P, namely more complete tumoricidal effect on implanted R1-tumors *vs* variable outcomes in primary HCCs, simultaneously occurring in the same rats with cirrhotic livers, which could help to guide future clinical applications of VDAs.

ARTICLE HIGHLIGHTS

Research background
Previously, all favorable responses to the vascular disrupting agent (VDA) combretastatin-A4-phosphate (CA4P) on implanted liver tumors were derived from animals with healthy liver. Yet, the diverse and paradoxical responses to CA4P on primary hepatomas have been from rats with cirrhotic liver.

Research motivation
Therapeutic responses of CA4P between primary and secondary hepatic tumors had never been compared intra-individually in the same rats with underlying liver cirrhosis. And, the potential microenvironmental impact from the surrounding liver parenchyma needed to be assessed further.

Research objectives
We aimed to compare therapeutic responses of CA4P among carcinogen-induced primary hepatocellular carcinomas (HCCs) and surgically implanted rhabdomyosarcoma (R1) in the same rats by magnetic resonance imaging (MRI), microangiography and histopathology.

Research methods
We performed diethylnitrosamine gavage to induce primary HCCs and simultaneous intrahepatic implantation of R1 to create secondary liver tumor in the same rats. Tumor growth was monitored by T2-/T1-weighted images on a 3.0T MRI scanner. Rats were then intravenously treated with CA4P. Vascular response and tumoral necrosis before and after treatment were compared by dynamic contrast-enhanced (DCE-) and CE-MRI. Tumor blood supply was further calculated by a semiquantitative DCE parameter of area under the time signal intensity curve (AUC30). Eventually, *in vivo* MRI findings were validated by postmortem techniques.

Research results
In total, 19 primary HCCs and 7 hepatic R1 allografts were successfully established in the 7 rats of the CA4P group, while 17 primary HCCs and 7 R1-tumors were generated in the 7 rats of the sham group. Uniform and variable vascularity were identified, respectively, in hepatic R1 allografts and primary HCCs. As documented by *in vivo* MRI and postmortem histopathology, vascular shutdown generally occurred at 1 h after CA4P treatment; at 12 h after treatment, tumoricidal effects were observed in secondary R1 tumors, while heterogeneous responses were seen in the primary HCCs. Quantitatively, tumor blood supply reflected by AUC30 showed vascular closure (66%) in R1-tumors at 1 h ($P < 0.05$), followed by further perfusion decrease at 12 h ($P < 0.01$); less significant vascular clogging occurred in HCCs. Histomorphologically, CA4P induced more extensive necrosis in R1-tumors (92.6%) than in HCCs (50.2%) ($P < 0.01$); tumor vascularity heterogeneously scored +~+++ in HCCs but homogeneously scored ++ in R1-tumors.

Research conclusions
To verify our original hypothesis that primary and secondary liver cancers may respond differently to VDA therapy due to the dissimilar tumor vascularity, a complex rat tumor model combining carcinogen-induced primary HCCs

and a surgically implanted R1-tumor in the same cirrhotic rats has thus been established to compare CA4P therapeutic responses intra-individually under the same microenvironment. Indeed, our hypothesis was verified by the superior performance of CA4P in metastatic over primary liver cancers. This could help to design future clinical trials and guide applications of VDAs.

Research perspectives

The merit of this study is that the present synchronous multiple liver cancer model in rodents could be a stepping-stone to help predict the diverse responses that may occur in patients, and to further address more complicated clinically relevant questions. The lesson that could be learnt from this study lies in the fact that although HCCs are generally hypervascularized, we should not take it for granted that the rich abnormal blood vessels naturally serve as plentiful drug targets for the VDA to inevitably induce massive tumor necrosis. This preclinical study's findings help in preparing a novel dual targeting pan-anticancer theragnostic strategy OncoCiDia in human liver cancers where CA4P could be applied as the first step.

REFERENCES

1   **Tozer GM**, Kanthou C, Baguley BC. Disrupting tumour blood vessels. *Nat Rev Cancer* 2005; **5**: 423-435 [PMID: 15928673 DOI: 10.1038/nrc1628]

2   **Cooney MM**, van Heeckeren W, Bhakta S, Ortiz J, Remick SC. Drug insight: vascular disrupting agents and angiogenesis--novel approaches for drug delivery. *Nat Clin Pract Oncol* 2006; **3**: 682-692 [PMID: 17139319 DOI: 10.1038/ncponc0663]

3   **Siemann DW**, Chaplin DJ, Walicke PA. A review and update of the current status of the vasculature-disabling agent combretastatin-A4 phosphate (CA4P). *Expert Opin Investig Drugs* 2009; **18**: 189-197 [PMID: 19236265 DOI: 10.1517/13543780802691068]

4   **Zweifel M**, Jayson GC, Reed NS, Osborne R, Hassan B, Ledermann J, Shreeves G, Poupard L, Lu SP, Balkissoon J, Chaplin DJ, Rustin GJ. Phase II trial of combretastatin A4 phosphate, carboplatin, and paclitaxel in patients with platinum-resistant ovarian cancer. *Ann Oncol* 2011; **22**: 2036-2041 [PMID: 21273348 DOI: 10.1093/annonc/mdq708]

5   **Sosa JA**, Elisei R, Jarzab B, Bal CS, Koussis H, Gramza AW, Ben-Yosef R, Gitlitz BJ, Haugen B, Karandikar SM, Khuri FR, Licitra LF, Remick SC, Marur S, Lu C, Ondrey FG, Lu S, Balkissoon J. A randomized phase II/III trial of a tumor vascular disrupting agent fosbretabulin tromethamine (CA4P) with carboplatin (C) and paclitaxel (P) in anaplastic thyroid cancer (ATC): Final survival analysis for the FACT trial. *J Clin Oncol* 2011; **29**: 5502–5502 [DOI: 10.1200/jco.2011.29.15_suppl.5502]

6   **Garon EB**, Kabbinavar FF, Neidhart JA, Neidhart JD, Gabrail NY, Oliveira MR, Lu SP, Balkissoon J. Randomized phase II trial of a tumor vascular disrupting agent fosbretabulin tromethamine (CA4P) with carboplatin (C), paclitaxel (P), and bevacizumab (B) in stage IIIb/IV nonsquamous non-small cell lung cancer (NSCLC): The FALCON trial. *J Clin Oncol* 2010; **28**: 7587–7587 [DOI: 10.1200/jco.2010.28.15_suppl.7587]

7   **He X**, Li S, Huang H, Li Z, Chen L, Ye S, Huang J, Zhan J, Lin T. A pharmacokinetic and safety study of single dose intravenous combretastatin A4 phosphate in Chinese patients with refractory solid tumours. *Br J Clin Pharmacol* 2011; **71**: 860-870 [PMID: 21276042 DOI: 10.1111/j.1365-2125.2011.03928.x]

8   **Grosios K**, Holwell SE, McGown AT, Pettit GR, Bibby MC. In vivo and in vitro evaluation of combretastatin A-4 and its sodium phosphate prodrug. *Br J Cancer* 1999; **81**: 1318-1327 [PMID: 10604728 DOI: 10.1038/sj.bjc.6692174]

9   **Wang H**, Li J, Chen F, De Keyzer F, Yu J, Feng Y, Nuyts J, Marchal G, Ni Y. Morphological, functional and metabolic imaging biomarkers: assessment of vascular-disrupting effect on rodent liver tumours. *Eur Radiol* 2010; **20**: 2013-2026 [PMID: 20182730 DOI: 10.1007/s00330-010-1743-5]

10 **Li J**, Sun Z, Zhang J, Shao H, Cona MM, Wang H, Marysael T, Chen F, Prinsen K, Zhou L, Huang D, Nuyts J, Yu J, Meng B, Bormans G, Fang Z, de Witte P, Li Y, Verbruggen A, Wang X,

Mortelmans L, Xu K, Marchal G, Ni Y. A dual-targeting anticancer approach: soil and seed principle. *Radiology* 2011; **260**: 799-807 [PMID: 21712473 DOI: 10.1148/radiol.11102120]

11 **Liu Y**, De Keyzer F, Wang Y, Wang F, Feng Y, Chen F, Yu J, Liu J, Song S, Swinnen J, Bormans G, Oyen R, Huang G, Ni Y. The first study on therapeutic efficacies of a vascular disrupting agent CA4P among primary hepatocellular carcinomas with a full spectrum of differentiation and vascularity: correlation of MRI-microangiography-histopathology in rats. *Int J Cancer* 2018 (accepted)

12 **Liu Y**, Yin T, Keyzer F, Feng Y, Chen F, Liu J, Song S, Yu J, Vandecaveye V, Swinnen J, Bormans G, Himmelreich U, Oyen R, Zhang J, Huang G, Ni Y. Micro-HCCs in rats with liver cirrhosis: paradoxical targeting effects with vascular disrupting agent CA4P. *Oncotarget* 2017; **8**: 55204-55215 [PMID: 28903414 DOI: 10.18632/oncotarget.19339]

13 **Liu Y**, Yin T, Feng Y, Cona MM, Huang G, Liu J, Song S, Jiang Y, Xia Q, Swinnen JV, Bormans G, Himmelreich U, Oyen R, Ni Y. Mammalian models of chemically induced primary malignancies exploitable for imaging-based preclinical theragnostic research. *Quant Imaging Med Surg* 2015; **5**: 708-729 [PMID: 26682141 DOI: 10.3978/j.issn.2223-4292.2015.06.01]

14 **Ni Y**, Marchal G, Yu J, Mühler A, Lukito G, Baert AL. Prolonged positive contrast enhancement with Gd-EOB-DTPA in experimental liver tumors: potential value in tissue characterization. *J Magn Reson Imaging* 1994; **4**: 355-363 [PMID: 8061434 DOI: 10.1002/jmri.1880040322]

15 **Ni Y**, Wang H, Chen F, Li J, DeKeyzer F, Feng Y, Yu J, Bosmans H, Marchal G. Tumor models and specific contrast agents for small animal imaging in oncology. *Methods* 2009; **48**: 125-138 [PMID: 19328231 DOI: 10.1016/j.ymeth.2009.03.014]

16 **Tozer GM**, Kanthou C, Lewis G, Prise VE, Vojnovic B, Hill SA. Tumour vascular disrupting agents: combating treatment resistance. *Br J Radiol* 2008; **81**: S12-S20 [PMID: 18819993 DOI: 10.1259/bjr/36205483]

17 **Wu XY**, Ma W, Gurung K, Guo CH. Mechanisms of tumor resistance to small-molecule vascular disrupting agents: treatment and rationale of combination therapy. *J Formos Med Assoc* 2013; **112**: 115-124 [PMID: 23473523 DOI: 10.1016/j.jfma.2012.09.017]

18 **Li J**, Chen F, Feng Y, Cona MM, Yu J, Verbruggen A, Zhang J, Oyen R, Ni Y. Diverse responses to vascular disrupting agent combretastatin a4 phosphate: a comparative study in rats with hepatic and subcutaneous tumor allografts using MRI biomarkers, microangiography, and histopathology. *Transl Oncol* 2013; **6**: 42-50 [PMID: 23418616 DOI: 10.1593/tlo.12367]

19 **Yin T**, Liu Y, Peeters R, Feng Y, Yu J, Himmelreich U, Oyen R, Ni Y. Vascular disrupting agent in pancreatic and hepatic tumour allografts: observations of location-dependent efficacy by MRI, microangiography and histomorphology. *Br J Cancer* 2017; **117**: 1529-1536 [PMID: 28910821 DOI: 10.1038/bjc.2017.324]

20 **Wang H**, Van de Putte M, Chen F, De Keyzer F, Jin L, Yu J, Marchal G, de Witte P, Ni Y. Murine liver implantation of radiation-induced fibrosarcoma: characterization with MR imaging, microangiography and histopathology. *Eur Radiol* 2008; **18**: 1422-1430 [PMID: 18343928 DOI: 10.1007/s00330-008-0904-2]

21 **Wang H**, Sun X, Chen F, De Keyzer F, Yu J, Landuyt W, Vandecaveye V, Peeters R, Bosmans H, Hermans R, Marchal G, Ni Y. Treatment of rodent liver tumor with combretastatin a4 phosphate: noninvasive therapeutic evaluation using multiparametric magnetic resonance imaging in correlation with microangiography and histology. *Invest Radiol* 2009; **44**: 44-53 [PMID: 19034028 DOI: 10.1097/RLI.0b013e31818e5ace]

22 **Maida M**, Macaluso FS, Galia M, Cabibbo G. Hepatocellular carcinoma and synchronous liver metastases from colorectal cancer in cirrhosis: A case report. *World J Hepatol* 2013; **5**: 696-700 [PMID: 24409337 DOI: 10.4254/wjh.v5.i12.696]

23 **Tofts PS**, Berkowitz BA. Rapid measurement of capillary permeability using the early part of the dynamic Gd-DTPA MRI enhancement curve. *J Magn Reson B* 1993; **102**: 129–136 [DOI: 10.1006/jmrb.1993.1075]

24   **Yankeelov TE**, Gore JC. Dynamic Contrast Enhanced Magnetic Resonance Imaging in Oncology: Theory, Data Acquisition, Analysis, and Examples. *Curr Med Imaging Rev* 2009; **3**: 91-107 [PMID: 19829742 DOI: 10.2174/157340507780619179]

25   **Buijs M**, Vossen JA, Geschwind JF, Salibi N, Pan L, Ventura VP, Liapi E, Lee KH, Kamel IR. Quantitative proton MR spectroscopy as a biomarker of tumor necrosis in the rabbit VX2 liver tumor. *J Vasc Interv Radiol* 2011; **22**: 1175-1180 [PMID: 21620723 DOI: 10.1016/j.jvir.2011.03.016]

26   **Schlageter M**, Terracciano LM, D'Angelo S, Sorrentino P. Histopathology of hepatocellular carcinoma. *World J Gastroenterol* 2014; **20**: 15955-15964 [PMID: 25473149 DOI: 10.3748/wjg.v20.i43.15955]

27   **Ni Y**, Marchal G, van Damme B, van Hecke P, Michiels J, Zhang X, Yu J, Baert AL. Magnetic resonance imaging, microangiography, and histology in a rat model of primary liver cancer. *Invest Radiol* 1992; **27**: 689-697 [PMID: 1399452 DOI: 10.1097/00004424-199209000-00006]

28   **Folkman J**, Cotran R. Relation of vascular proliferation to tumor growth. *Int Rev Exp Pathol* 1976; **16**: 207-248 [PMID: 783062]

29   **Donnem T**, Hu J, Ferguson M, Adighibe O, Snell C, Harris AL, Gatter KC, Pezzella F. Vessel co-option in primary human tumors and metastases: an obstacle to effective anti-angiogenic treatment? *Cancer Med* 2013; **2**: 427-436 [PMID: 24156015 DOI: 10.1002/cam4.105]

30   **Bugyik E**, Renyi-Vamos F, Szabo V, Dezso K, Ecker N, Rokusz A, Nagy P, Dome B, Paku S. Mechanisms of vascularization in murine models of primary and metastatic tumor growth. *Chin J Cancer* 2016; **35**: 19 [PMID: 26873579 DOI: 10.1186/s40880-016-0083-5]

31   **Paku S**, Kopper L, Nagy P. Development of the vasculature in "pushing-type" liver metastases of an experimental colorectal cancer. *Int J Cancer* 2005; **115**: 893-902 [PMID: 15729725 DOI: 10.1002/ijc.20886]

32   **Yang ZF**, Poon RT. Vascular changes in hepatocellular carcinoma. *Anat Rec* (Hoboken) 2008; **291**: 721-734 [PMID: 18484619 DOI: 10.1002/ar.20668]

33   **Maier KP**. [Cirrhosis of the liver as a precancerous condition]. *Praxis* (Bern 1994) 1998; **87**: 1462-1465 [PMID: 9847685]

34   **Schuppan D**, Afdhal NH. Liver cirrhosis. *Lancet* 2008; **371**: 838-851 [PMID: 18328931 DOI: 10.1016/S0140-6736(08)60383-9]

35   **Li J**, Cona MM, Chen F, Feng Y, Zhou L, Yu J, Nuyts J, de Witte P, Zhang J, Himmelreich U, Verbruggen A, Ni Y. Exploring theranostic potentials of radioiodinated hypericin in rodent necrosis models. *Theranostics* 2012; **2**: 1010-1019 [PMID: 23139728 DOI: 10.7150/thno.4924]

36   **Ni Y**. Abstract 1767: Oncocidia: a small molecule dual targeting pan-anticancer theragnostic strategy. *Cancer Res* 2014; **74**: 1767–1767 [DOI: 10.1158/1538-7445.AM2014-1767]

ORIGINAL ARTICLE

Retrospective Study

Gastric cancer in Alaska Native people: A cancer health disparity

Holly A Martinson, Nancy J Shelby, Steven R Alberts, Matthew J Olnes

Holly A Martinson, Nancy J Shelby, WWAMI School of Medical Education, University of Alaska Anchorage, Anchorage, AK 99508, United States

Steven R Alberts, Department of Oncology, Mayo Clinic Cancer Center, Rochester, MN 55905, United States

Matthew J Olnes, Hematology and Medical Oncology Department, Alaska Native Medical Center, Anchorage, AK 99508, United States

ORCID number: Holly A Martinson (0000-0003-2745-2755); Nancy J Shelby (0000-0002-4069-0937); Steven R Alberts (0000-0002-5835-4122); Matthew J Olnes (0000-0002-7791-5554).

Abstract

AIM

To evaluate recent trends in gastric cancer incidence, response to treatment, and overall survival among Alaska Native (AN) people.

METHODS

A retrospective analysis of the Alaska Native Medical Center patient database was performed. Patient history, clinical, pathological, response to treatment and patient outcomes were collected from one-hundred and thirty-

two AN gastric cancer patients. The Surveillance, Epidemiology and End Result database 18 was used to collect comparison United States non-Hispanic White (NHW) and AN gastric cancer patient data between 2006-2014.

RESULTS
AN gastric cancer patients have a higher incidence rate, a poorer overall survival, and are diagnosed at a significantly younger age compared to NHW patients. AN patients differ from NHW patients in greater prevalence of non-cardia, diffuse subtype, and signet ring cell carcinomas. AN females were more likely to be diagnosed with later stage cancer, stage IV, compared to AN males. Diminished overall survival was observed among AN patients with increasing stage, O+ blood type, < 15 lymph nodes examined at resection, and no treatment. This study is the first report detailing the clinicopathologic features of gastric cancer in AN people with outcome data.

CONCLUSION
Our findings confirm the importance of early detection, treatment, and surgical resection for optimizing AN patient outcomes. Further research on early detection markers are warranted.

Core tip: Gastric cancer (GC) is a leading cancer health disparity among the Alaska Native (AN) people. The aim of this study was to evaluate recent trends in AN gastric cancer incidence and survival. AN patients differ from non-Hispanic White patients in increased incidence, younger age at diagnosis, a higher presence of non-cardia, diffuse subtype, signet ring cell carcinomas, *Helicobacter pylori*, and greater proportion of GC among women. AN patients diagnosed at an early stage and whom receive surgical treatment have better overall survival compared to later stage patients. Therefore, additional screening programs and early detection measures for AN people, may improve patient outcomes.

INTRODUCTION

Worldwide, there are significant racial disparities in incidence and mortality of gastric cancer. The highest incidence and mortality rates occur in Eastern Asia, Eastern Europe, Central America, and South America

populations, whereas North America and Africa populations have the lowest incidence[1,2]. In recent years, gastric cancer incidence has declined in Eastern Asia and Asian patients have been shown to have better outcomes compared to other ethnic groups such as in South American, United States Hispanic, and African American patients[2,3]. In the United States (US), gastric cancer incidence rates are also declining in the general population[4] and recently in racial ethnic subgroups such as Asian Americans who have had a historically high prevalence of gastric cancer[5]. In contrast, gastric cancer incidence and mortality rates have remained the same among the Alaska Native (AN) population, becoming one of the leading cancer health disparities[6-8].

The AN population has a 3-fold higher incidence and mortality rate of gastric cancer when compared to the US non-Hispanic White (NHW) population[6,7,9]. AN patients are often diagnosed with advanced stage disease, and have a poor overall 5-year survival rate of less than 20%[10,11]. Gastric cancer etiology differs between the NHW and AN populations. NHW patients are most often diagnosed with gastric cancers in the cardia, gastroesophageal junction or distal esophagus, while gastric cancers in AN patients are localized to the central and distal stomach[9,12]. Further, differences in tumor subtype have been observed between the two populations, with the diffuse subtype being most common among AN patients[7]. The high incidence and prevalence of non-cardia gastric cancers among AN patients has been associated with the high seropositivity rates of *Helicobacter pylori* (*H. pylori*) among the general AN population[13-15]. In addition to *H. pylori*, multiple risk factors could contribute to this cancer health disparity among the AN people, including socioeconomic factors and biological differences, such as access to treatment, genetic influences, lifestyle differences, and environmental exposures.

A greater understanding of gastric cancer incidence and response to treatment among the AN people may allow for the design of screening programs or the identification of early detection measures to potentially reduce incidence and improve patient outcomes. In order to further investigate how to reduce gastric cancer incidence and mortality rates among the AN population, we sought to evaluate recent trends in gastric cancer incidence, as well as to report on clinical response to treatment and overall survival outcomes in this high incidence population.

MATERIALS AND METHODS

Alaska Native gastric cancer patients
The University of Alaska Anchorage Institutional Review Board (IRB), Alaska Area IRB, Southcentral Foundation Review Board, and the Alaska Native Tribal Health Consortium Health Research Review Committee approved this study. The medical records were reviewed from one-hundred and thirty-two AN patients

with histologically confirmed gastric adenocarcinoma presenting at the Alaska Native Medical Center (ANMC), a referral hospital for the Alaska Tribal Health System, from January 2006 to December 2014. Demographic and clinicopathologic variables obtained from medical records included: sex, age at diagnosis, region of Alaska where patient resides, tumor grade, stage, primary location, metastatic site, histologic type according to Lauren classification[16], histological appearance, stage, type of therapy, order of treatment, surgical resection, lymph nodes examined, recurrence site, overall survival, presence of *H. pylori* at time of biopsy/resection, chronic gastritis, gastroesophageal reflux disease (GERD), gastric ulcer, blood type, self-reported family history of gastrointestinal cancers and tobacco use. Patients were classified into five regions: far north, interior, southwest, southcentral, and southeast designated by the Alaska Department of Labor and Workforce Development 2010 census. Vital status was obtained through Social Security Disability Insurance program or through the Alaska Department of Health and Social Services.

SEER database

Data collected on US NHW and AN gastric adenocarcinoma patients were obtained from the US National Institute's SEER Program of the National Cancer Institute 18 dataset for the period 2006-2014. The SEER program collects information on incidence, prevalence, survival, and cancer mortality from cancer registries representing approximately 28% of the US population. The SEER database captures all cancer cases among the AN population, approximately 150000 people, through the Alaska Native Tumor Registry. SEER*Stat software (www.seer.cancer.gov/seerstat) Version 8.2.1 was used for analysis of data.

Data classification and coding

Overall survival was calculated from the date of gastric cancer diagnosis until death from any cause or date of last follow-up. Patient vital status was confirmed through the ANMC tumor registry. Survival times of patients with stable disease were censored at the last follow-up date. Anatomical subsite and histological conditions were grouped using the International Classification of Disease for Oncology 3 (ICD-O-3) codes. ICD-O-3 codes used in the study were: 8140 Adenocarcinoma, not otherwise specified (NOS); 8142, Linitis plastica; 8144/3, adenocarcinoma, intestinal type; 8145/3, adenocarcinoma, diffuse type; 8211/3, tubular adenocarcinoma; 8255/3, adenocarcinoma with mixed subtypes; 8260/3, papillary adenocarcinoma, NOS; 8480/3, mucinous adenocarcinoma; and 8490/3, signet ring carcinoma. For anatomic subsite analysis the four-digit topography site codes (C15, esophageal cancer and C16, stomach cancer) were used to extract and analyze the incident cases of gastric cancer. Four anatomic subsites were formed: Cardia, C15.5 lower third esophagus and C16.0 cardia; Non-Cardia, C16.1 fundus, C16.2 body, C16.3

gastric antrum, C16.4 pylorus, C16.5 lesser curvature of stomach NOS, C16.6 greater curvature of stomach NOS; Overlapping, C16.8 overlapping lesion of stomach; and Unspecified. C16.9 stomach NOS. Pathological stage was classified according to the American Joint Committee on Cancer (AJCC) 7th edition manual for stomach cancer[17]. The number of lymph nodes examined following resection were dichotomized into < 15 or ≥ 15 based on recommendation from the National Comprehensive Cancer Network[18].

Statistical analysis

Raw frequencies and percentages of cases for available data from the US SEER database and the ANMC hospital are reported. Data were analyzed using software SPSS 23.0 (SPSS Inc, Chicago, IL, United States). Patient demographics and clinicopathological characteristics between NHW versus AN and AN male versus AN female were compared using chi-square tests for categorical variables and Student t tests for continuous variables. Wilcoxon rank-sum (Mann-Whitney) test was used for variables that were not normally distributed. Association between various clinicopathological characteristics and overall survival were examined with Cox proportional hazard models. The Kaplan-Meier method was used for survival analysis, and differences in survival between groups were evaluated using the log-rank test. Variables with a P value < 0.1 on univariate analysis were included in the multivariate Cox proportional hazards regression model analysis. A two-sided P value of < 0.05 was considered significant.

RESULTS

Gastric cancer in AN people is distinct from non-Hispanic white people

Between 2006-2014, a total of 132 AN patients with adenocarcinoma of the gastroesophageal junction or stomach were identified from the ANMC hospital database. In the same period of time using the US SEER database, we identified 40717 NHW patients and 164 AN patients with adenocarcinoma of the gastroesophageal junction or stomach. Similar trends were observed between the AN Hospital and AN SEER data. As shown in Table 1, there were significant differences in the clinicopathological characteristics between the NHW and AN patients. Compared to the US NHW population, AN patients (AN SEER and AN Hospital) have a higher incidence rate and were significantly younger at time of diagnosis (59.9 years *vs* 69.2 years; $P < 0.0001$) (Figure 1). Also, the AN patients had significant differences in tumor location and appearance, with a higher prevalence of non-cardia tumors (60.6% *vs* 21.3%; $P < 0.0001$, Table 1) and signet ring cell carcinomas (39.4% *vs* 12.7%; $P < 0.0001$). AN patients were significantly more likely to be diagnosed with stage IV disease (50.0% *vs* 37.6%; $P = 0.03$, Table 1). We also observed among the AN patients a larger proportion of females (37.8%

Table 1 Comparative epidemiology of gastric cancer in United States Non-Hispanic White and Alaska Native populations *n* (%)

	United States			P[1] value
	Non-Hispanic White	**Alaska Native-SEER**	**Alaska Native Hospital**	
Population (in millions)[2,3]	274.6	0.14	0.14	
Cancer registry summary				
Registry type (number)	Population based (SEER 18)	Population based (SEER 18)	Hospital based (1)	
Years included	2006-2014	2006-2014	2006-2014	
Incident cases	40717	164	132	
Calculated incidence rate[4]				
Male	12.1	26.7	22.8	
Female	3.4	18.7	13.6	
Gender				**0.01**
Male	30141 (74.0)	95 (57.9)	82 (62.1)	
Female	10576 (26.0)	69 (42.1)	50 (37.8)	
Age				**< 0.0001[5]**
Median (yr)	69.2 ± 0.07	60.4 ± 1.3	59.9 ± 1.2	
< 20-34	258 (0.6)	4 (2.4)	3 (2.3)	
35-44	926 (2.3)	9 (5.5)	9 (6.8)	
45-54	4150 (10.2)	44 (26.8)	39 (29.5)	
55-64	9224 (22.7)	41 (25.0)	28 (21.2)	
65-74	11089 (27.2)	38 (23.2)	30 (22.7)	
75-84	10144 (24.9)	23 (14.0)	19 (14.4)	
> 84	4927 (12.1)	5 (3.0)	4 (3.0)	
Anatomic site				**< 0.0001[7]**
Cardia	26387 (64.8)	38 (23.2)	22 (16.7)	
GE JX	14502 (35.6)	14 (8.5)	12 (9.1)	
Non-cardia	8659 (21.3)	86 (52.4)	80 (60.6)	
Fundus	970 (2.4)	4 (2.4)	12 (9.1)	
Body[6]	3726 (9.2)	52 (30.0)	33 (25.0)	
Antrum	3483 (8.6)	22 (13.4)	18 (13.6)	
Pylorus	480 (1.2)	11 (6.7)	17 (12.9)	
Overlap (multifocal)	1523 (3.7)	9 (5.5)	27 (20.5)	
Unspecified	4148 (10.2)	31 (18.9)	3 (2.3)	
Histological appearance				**< 0.0001[8]**
Adenocarcinoma, NOS	33,921 (83.3)	126 (76.8)	80 (60.6)	
Linitis Plastica, AC	172 (0.4)	1 (0.6)	2 (1.5)	
Mucinous, AC	626 (1.5)	3 (1.8)	5 (3.8)	
Tubular, AC	168 (0.5)	1 (0.6)	1 (0.8)	
Papillary, AC	91 (0.2)	1 (0.6)	1 (0.8)	
Mixed Cell, AC	560 (1.4)	1 (0.6)	0 (0)	
Signet Ring	5179 (12.7)	31 (18.9)	52 (39.4)	
Stage				**0.002**
I	8901 (21.9)	35 (21.3)	28 (21.1)	
II	5662 (13.9)	24 (14.6)	24 (18.0)	
III	5327 (13.1)	14 (8.5)	14 (11.4)	
IV	15323 (37.6)	75 (45.7)	66 (50.0)	
Unspecified	5504 (13.5)	16 (9.8)	0 (0)	

[1]Bold type indicates statistical significance ($P < 0.05$), statistics were performed on SEER Non-Hispanic White and Hospital based Alaska Native populations; [2]Population for US White 2010, US Census; [3]Population for AK Native 2010, Alaska Department of Labor and workforce development; [4]Incidence rates, per 100000 person years and are age-adjusted using the 2000 US standard population; [5]Wilcoxon rank sum test; [6]Body includes body, lesser curvature, and greater curvature; [7]Chi-square test between Cardia, Non-Cardia, Overlapping, and Unspecified; [8]Chi-square test between adenocarcinoma and Signet Ring. NOS: Not otherwise specified; AC: Adenocarcinoma; GE JX: Gastroesophageal junction; SEER: Surveillance and Epidemiology End Results Program (SEER) 18 dataset; AC: Adenocarcinoma; JX: Junction.

vs 26.0%; *P* = 0.01, Table 1). AN females had a higher prevalence of signet ring cell carcinoma compared to NHW females (46% *vs* 35.4%).

Epidemiology and clinical features of gastric cancer in AN people

The median age of AN cancer patients was 58.3 years for men and 62.4 years for women (Table 2). AN males diagnosed with gastric cancer had significantly higher rates of tobacco use compared to females (87.8% *vs* 72.0% *P* = 0.04). In addition, we observed gastric cancer in AN males were more likely to metastasize to multiple sites compared to females (29.4% *vs* 18.8%). AN female and male patients did not differ in their geographical location, of which the majority of patients resided in three regions of Alaska: North (28.8%), Southwest (30.3%), and Southcentral (30.3%) (Table 2). AN females were more likely to be diagnosed with stage IV gastric cancer (64% vs 41.5%) and choose not to seek treatment (36% *vs* 24.4% respectively) compared to AN males. AN male and female gastric patients had similar distribution for blood type, grade,

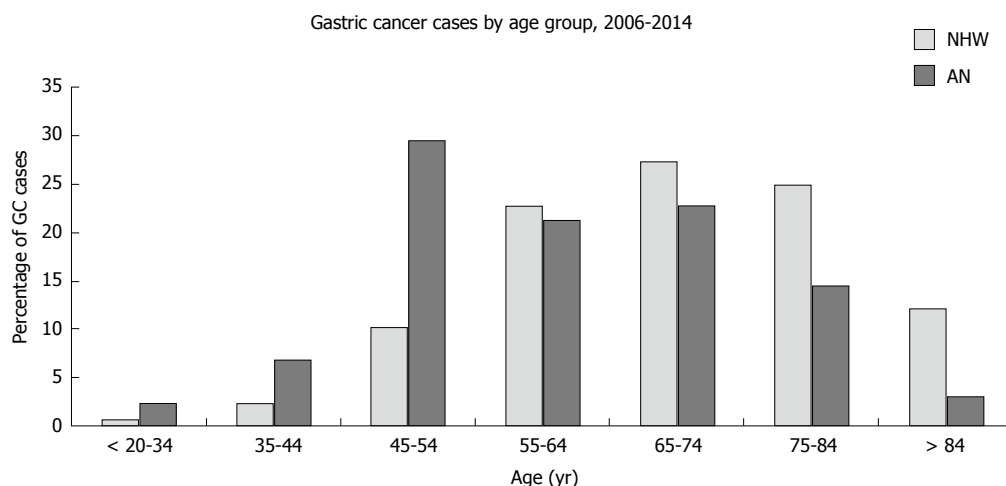Gastric cancer cases by age group, 2006-2014

Figure 1 Age group distribution for non-Hispanic White and Alaska Native patients diagnosed with gastric cancer 2006-2014. AN: Alaska Native; NHW: Non-Hispanic White.

primary tumor location, treatment, *H. pylori* positive tumor, chronic gastritis, GERD, gastric ulcers, and family history of gastric and/or colorectal cancer.

Clinical response to treatment and overall survival in AN gastric cancer patients
Results of univariate Cox proportional regression analysis for factors associated with overall survival are shown in Table 3. In this study, 109 deaths (82.6%) were observed among the AN patients (*n* = 132) within a five-year study period with greater than 90% of patients dying from gastric cancer. Anatomic site, AJCC stage, treatment, the number of lymph nodes examined during resection, and blood type were associated with diminished overall survival. Patients with tumors diagnosed at stage IV, and poorly/undifferentiated histology had a higher risk of death. Patients whose tumors involved multiple regions of the stomach (overlap), upper third, and GE junction had decreased survival times when compared to patients with distal tumors. Patients with O+ blood type had significantly poorer survival compared to A+ and AB+ patients (Table 3). Patients were treated with chemotherapy, surgery, and radiation according to standard NCCN guidelines[18]. Those who received a gastric resection with or without additional therapy had significantly better survival compared to patients that received only chemotherapy. Patients who received neoadjuvant chemotherapy with or without radiation, followed by surgery, and adjuvant chemotherapy had the best survival.

In univariate analysis, patients who were diagnosed with chronic gastritis at the time of gastric cancer diagnosis had better survival than people without gastritis (Table 3). Upon further investigation, patients were more likely to be diagnosed with stage IV cancer without gastritis (75%) compared to patients with chronic gastritis (43%). We did not observe differences in survival in patients who had *H. pylori* positive tumors, the presence of signet ring cells, diffuse type tumors,

or gastric ulcers at the time of diagnosis. The results of the multivariable analysis for association with overall survival are shown in Table 4. This analysis revealed that the variables independently associated with overall survival included AJCC stage and treatment modality, with neoadjuvant chemotherapy or chemoradiation followed by surgery and adjuvant therapy having the greatest association with overall survival.

DISCUSSION

Evaluating the clinicopathological features of gastric adenocarcinoma in AN patients, we found significant diverse outcomes in epidemiological factors and survival outcomes compared to NHW patients. Our study showed that age-adjusted incidence rates were higher among AN patients compared to US NHW patients. Further, AN patients were more likely to be younger at time of diagnosis, develop non-cardia gastric cancer, and have gastric cancers with signet ring cell histological features. These findings correlate to what has been observed in gastric cancer patients in developing countries and in Asian American populations[3,5]. However, gastric cancer patients in developing countries and in the US tend to be more often male (3:1 male to female), which was not seen represented in our patient population (1.6:1 male to female). A male to female sex ratio of 1:1 has been reported amongst young NHW gastric cancer patients[19,20] and more recent data indicates non-cardia cancers are on the rise in patients younger than 50, particularly among females[21]. A unique observation from our study was that AN females had a higher rate of signet ring cell carcinoma, compared to NHW patients and AN males. High incidence of signet ring cell carcinoma has been reported in other ethnic groups (African American, Asian, AI/AN, and Hispanic) as well as in female patients[22,23], however, the details of these associations have not been well investigated.

The younger age at diagnosis among AN patients

Table 2 Descriptive epidemiology of Alaska Native gastric cancer patients *n* (%)

	Overall	Female	Male
Patients	132	50 (37.9)	82 (62.1)
Mean age (yr)	59.8 ± 1.2	62.4 ± 2.0	58.2 ± 1.4
Histological type			
Diffuse	75 (56.8)	29 (58.0)	46 (56.1)
Intestinal	51 (38.6)	16 (32.0)	35 (42.7)
NOS	6 (4.5)	5 (10.0)	1 (1.2)
Blood type			
A+	55 (41.7)	23 (46.0)	32 (39.0)
AB+	8 (6.1)	3 (6.0)	5 (6.1)
B+	9 (6.8)	5 (10.0)	4 (4.9)
O+	30 (22.7)	10 (20.0)	20 (24.4)
Unknown	30 (22.7)	9 (18.0)	21 (25.6)
Histological appearance			
Signet Ring	52 (39.4)	23 (46.0)	29 (35.4)
Adenocarcinoma	80 (59.8)	27 (54.0)	53 (64.6)
Linitis Plastica, AC	2 (2.5)	0 (0)	2 (3.8)
Mucinous, AC	2 (2.5)	0 (0)	2 (3.8)
Tubular, AC	1 (1.3)	0 (0)	1 (1.9)
Papillary	1 (1.3)	0 (0)	1 (1.9)
NOS, AC	75 (93.8)	27 (100)	46 (86.8)
Stage			
I	28 (21.2)	8 (16.0)	20 (24.4)
II	24 (18.2)	6 (12.0)	18 (22.0)
III	14 (11.4)	4 (8.0)	10 (12.2)
IV	66 (50.0)	32 (64.0)	34 (41.5)
Grade			
Well/moderately differentiated	35 (26.5)	9 (18.0)	26 (31.7)
Poorly differentiated	88 (66.7)	36 (72.0)	52 (63.4)
Unknown	9 (6.8)	5 (10.0)	4 (4.9)
Anatomic site			
GE JX	12 (9.1)	5 (10.0)	7 (8.5)
Cardia	10 (7.6)	5 (10.0)	5 (6.1)
Fundus	12 (9.1)	6 (12.0)	6 (7.3)
Body	33 (25.0)	10 (20.0)	23 (28.0)
Antrum	18(13.6)	8 (16.0)	10 (12.2)
Pylorus	17 (12.9)	7 (14.0)	10 (12.2)
Overlap (multifocal)	27 (20.5)	9 (18.0)	18 (22.0)
Unspecified	3 (2.3)	0 (0)	3 (3.7)
Regions of Alaska			
North	38 (28.8)	17 (34.0)	21 (25.6)
Interior	11 (8.3)	1 (2.0)	10 (12.2)
Southwest	40 (30.3)	15 (30.0)	25 (30.5)
Southcentral	40 (30.3)	17 (34.0)	23 (28.0)
Southeast	3 (2.3)	0 (0)	3 (3.7)
Treatment[2]			
Chemotherapy only	40 (30.3)	12 (24.0)	28 (34.1)
Neoadjuvant	21 (15.9)	8 (16.0)	13 (15.9)
Adjuvant	12 (9.1)	4 (8.0)	8 (9.8)
Resection only	12 (9.1)	5 (10.0)	7 (8.5)
Neoadjuvant, resection, adjuvant	9 (6.8)	3 (6.0)	6 (7.3)
None	38 (28.8)	18 (36.0)	20 (24.4)
Metastasis site[1]			
Omentum/peritoneium/diaphram	27 (41.5)	14 (43.8)	13 (38.2)
Liver	14 (21.5)	6 (18.8)	8 (23.5)
Lung	1 (1.5)	0 (0)	1 (2.9)
Bone	2 (3.1)	0 (0)	2 (5.9)
Ovary	6 (9.2)	6 (18.8)	0 (0)
Multiple Sites	15 (23.1)	6 (18.8)	10 (29.4)
Additional clinical and pathological variables			
H. pylori	52 (41.3)	20 (41.7)	32 (41.0)
Chronic gastritis	101 (76.5)	38 (76.0)	63 (76.8)
GERD	51 (39.5)	18 (36.0)	33 (41.3)
Gastric ulcer	91 (68.9)	35 (70.0)	56 (68.3)
Tobacco	108 (81.8)	36 (72.0)	72 (87.8)
Family history GI cancer	38 (28.8)	16 (32.0)	21 (25.6)

[1]Patients with stage 4 gastric cancer were used for analysis; [2]Neoadjuvant and adjuvant treatments include chemotherapy and chemoradiation regimens based on NCCN guidelines. NOS: Not otherwise specified; AC: Adenocarcinoma; GE JX: Gastroesophageal junction; *H. pylori*: *Helicobacter pylori*; GERD: Gastroesophageal reflux disease; GC: Gastric cancer.

Table 3 Univariate analysis of Alaska Native gastric cancer patients to identify variables associated with overall survival

	HR[1]	95%CI	P[2] value
Age, ≥ 55 yr *vs* < 55 yr	0.73	0.49-1.07	0.11
Sex, male *vs* female	0.86	0.59-1.27	0.45
Geographical Region			0.53
North	1.00		
Interior	0.99	0.50-1.96	
Southwest	0.68	0.42-1.11	
Southcentral	1.00	0.61-1.62	
Southeast	0.65	0.15-2.69	
Signet ring, present or absent	0.78	0.52-1.17	0.22
Diffuse *vs* intestinal	1.01	0.68-1.49	0.97
Anatomic site			0.07
Noncardia Cardia	1.00		
Cardia[3]	1.10	0.66-1.84	
Overlap/NOS	1.74	1.08-2.81	
Grade			0.22
Poorly differentiated	1.00		
Well/moderately	0.70	0.66-1.84	
Unknown	0.82	0.37-1.84	
AJCC Stage			< 0.0001
I	1.00		
II	1.42	0.74-2.75	
III	1.67	0.79-3.54	
IV	4.91	2.80-8.61	
Treatment[4]			< 0.0001
Chemo	1.00		
None	2.07	1.29-3.32	
Neoadjuvant, surgery	0.23	0.12-0.45	
Surgery only	0.29	0.14-0.61	
Surgery, adjuvant	0.27	0.13-0.59	
Neoadjuvant, surgery, adjuvant	0.15	0.06-0.39	
No. of nodes examined, ≥ 15 *vs* < 15[5] nodes[3]	0.37	0.19-0.75	< 0.0001
Blood Type			0.04
A+	1.00		
AB+	0.88	0.42-1.82	
B+	1.72	0.76-3.96	
O+	1.78	1.05-3.01	
Unknown	2.35	1.24-4.45	
Multiple primaries, yes *vs* no	0.73	0.45-1.17	0.22
Tobacco			0.51
Yes	1.00		
None	0.83	0.49-1.42	
Chew/Iqmik	0.78	0.31-1.95	
Former user[6]	1.26	0.80-1.97	
Gastric ulcer, yes *vs* no	1.02	0.67-1.54	0.93
Chronic Gastritis, yes *vs* no	0.62	0.39-0.98	0.04
H. pylori, tumor positive *vs* negative	0.90	0.61-1.34	0.62
GERD, yes *vs* no	0.75	0.51-1.13	0.17

[1]Univariate analysis was performed using Kaplan-Meier analysis model and log-rank test; [2]Bold type indicates statistical significance (P < 0.05); [3]Cardia includes gastroesophageal junction and cardia gastric cancers; [4]Neoadjuvant and adjuvant treatments include chemotherapy and chemoradiation regimens based on NCCN guidelines; [5]Patients that had a resection were included in analysis; [6]Patients discontinued smoking before time of diagnosis. HR: Hazard ratio; CI: Confidence interval; AJCC: American Joint Committee on Cancer; Chemo: Chemotherapy; *H. pylori*: *Helicobacter pylori*; GERD: Gastroesophageal reflux disease.

with gastric cancer could be driven by multiple etiologies. One factor is earlier exposure to particular gastric cancer risk factors such as *H. pylori* infection and tobacco use. Previous research revealed 40% of AN children have been infected with *H. pylori* by age

4, 70% by age 10, and 78% by age 14[24]. This study and our results suggest the likelihood of long term exposure to systemic inflammation due to the early age of acquisition of *H. pylori* may play an important role in the high incidence of non-cardia cancer, younger age at diagnosis, and the overall gastric cancer health disparity among the AN people. Further, the high prevalence of tobacco use among the AN people may also contribute to the younger age of diagnosis of gastric cancer patients. Another variable associated with gastric cancer in younger individuals is genetic predisposition such as CDH1 germline mutations that result in hereditary diffuse gastric cancers. Approximately 30% of AN patients had a family history of gastrointestinal cancers and there was no difference in age of diagnosis. Further, other types of cancer among the AN people such as lung, kidney, and colorectal cancer are also associated with younger age of diagnosis suggesting earlier age of diagnosis of cancer is a general characteristic in AN cancer patients compared to NHW patients. Often cancers diagnosed at a younger age are more aggressive and are found at a later stage, which may also contribute to cancer health disparities among the AN people.

AN patients were more likely to be diagnosed with non-cardia gastric adenocarcinoma compared to NHW patients. Multiple epidemiological studies have shown non-cardia to be associated with other ethnic populations, such as Hispanics in Central America, US Hispanics, and Eastern Asians[4,25,26]. A commonality between these ethnic groups is the presence of *H. pylori* in non-cardia gastric cancer[27]. Of the AN patients, approximately half of them had active *H. pylori* infections at time of diagnosis, of which 65% of the positive cases were in patients diagnosed with non-cardia gastric cancer. However, it is unknown as to how many of the patients have been previously infected or treated for *H. pylori* during their lifetime. Fock *et al*[26] reported that the incidence of gastric adenocarcinoma in Asia tends to mirror the seroprevalence rate of *H. pylori* infection. The CDC has reported *H. pylori* seroprevalence rate of 75% among AN people[28], rates which are similar to or higher than the rates reported in Eastern Asia[26]. Further, the majority of *H. pylori* strains in the AN people are CagA and VacA positive[29], both of which are associated with an increased risk of developing severe gastritis, atrophic gastritis, peptic ulcer disease and distal gastric cancers[27,30-32]. Along with the high prevalence of *H. pylori*, both AN and Eastern Asians share a similar diet and lifestyle: High intake of salty and smoked foods, low vegetable intake and high rates of tobacco use, all of which may contribute to increased risk of gastric cancer[33]. Chronic inflammation and infection are of particular interest in the AN population due to the high levels of chronic gastritis, endemic rates of *H. pylori* infection, and an increased incidence of EBV-driven cancers in AN people, such as nasopharyngeal carcinoma and lymphoepithelial tumors of the parotid

Table 4 Multivariable Cox regression analysis for variables associated with overall survival

	HR	95%CI	*P*[1] value
AJCC stage			**0.004**
I	1.00		
II	1.59	0.74-3.32	
III	3.09	1.32-7.27	
IV	3.08	1.53-6.19	
Treatment			**0.007**
Chemotherapy	1.00		
None	2.80	1.57-5.00	
Neoadjuvant, surgery	0.28	0.10-5.31	
Surgery only	0.48	0.15-12.61	
Surgery, adjuvant	0.19	0.06-5.17	
Neoadjuvant, surgery, adjuvant	0.18	0.5-5.31	
Blood type			0.160
A+	1.00		
AB+	0.81	0.34-2.23	
B+	1.01	0.45-2.24	
O+	1.40	0.84-2.54	
Unknown	0.63	0.31-1.17	
Chronic gastritis, yes *vs* no	1.01	0.59-1.69	0.970

[1]Bold type indicates statistical significance (*P* < 0.05). HR: Hazard ratio; CI: Confidence interval; AJCC: American Joint Committee on Cancer.

gland[34,35]. Understanding how environmental and dietary factors play a role in increased risk of gastric cancer among AN people requires further investigation.

This study also revealed differences between AN male and female gastric cancer patients. AN males were diagnosed at an earlier age, while AN females were more likely to be diagnosed at a later stage. Many of AN female patients also elected not to receive treatment 36%, compared to 24% of males, regardless of stage of diagnosis. In our study, AN males were more likely to use tobacco compared to the AN females, which has been reported by Alaska's Behavioral Risk Factor Surveillance System (BRFSS) in the general AN population[36]. Further, 82% of AN patients were current or former smokers, which is higher than the reported 42% of the total AN population[36]. We also observed similar trends between AN males and females with regards to concurrent *H. pylori* infection, gastritis, GERD, and gastric ulcer. The diagnosis of gastritis, GERD, and gastric ulcers are all known risk factors for developing gastric cancer. Approximately a third of AN patients reported a family history of a first-degree relative with gastric or colorectal cancer, which is higher than reported in other studies[19,33,37]. Previous studies reported that gastric cancers within a population share similar pathological characteristics, suggesting the association of genetic, environmental and lifestyle factors with gastric carcinogenesis[7,38]. However, further research is needed in order to evaluate how genetic, environmental, and lifestyle factors play a role in AN gastric cancer.

Our study is the first study to evaluate clinical outcomes in AN gastric cancer patients. We observed that overall survival was influenced by AJCC stage, blood type, chronic gastritis and treatment. While

stage and treatment have been previously reported in the literature as significant prognostic factors of survival[4,37,39], blood type and chronic gastritis have never been associated with overall survival. AN patients with the blood group O had lower overall survival compared to the A/AB groups. Previous studies have reported that patients with blood type A are at a higher risk for developing gastric cancer[40-42]. AN patients with the presence of chronic gastritis were shown to have a more favorable prognosis, which was also associated with an earlier stage at diagnosis. This result suggests that AN patients presenting with symptoms of chronic gastritis may be at high risk for gastric cancer and may benefit from an endoscopy at time of initial presentation. There are currently no standard guidelines on screening for gastric cancer in the US[18], whereas Asian countries with a high incidence of gastric cancer have implemented screening programs using a variety of modalities. However, the most effective gastric cancer screening modality and the screening interval remains controversial. Furthermore, an improved understanding of the composition of immune cells present within the tumor and surrounding microenvironment as well as their function may further elucidate this observation.

AN patients are more likely to present with later stage disease resulting in worse outcomes and the inability of patients to obtain surgical resection, the only curative therapy for gastric cancer. The Alaska Tribal Health System is a unique health system with 58 tribal health centers, 160 tribal community health aide clinics, and 6 regional hospitals dispersed throughout a vast land mass that covers more than 25% of the contiguous US. Patients with cancer are referred to the Alaska Native Medical Center (ANMC), a tertiary hospital in Anchorage, Alaska where they receive cancer therapies according to standard international guidelines[18]. Many of the AN patients included in this study must travel to ANMC to receive their care and medical treatments. The average patient distance from ANMC and its effect on patient care and outcomes has not been studied but is worthy of further investigation. Our study revealed AN patients who received surgery with or without chemotherapy had a better overall survival compared to patients who received chemotherapy alone. Further, patients who received neoadjuvant, surgery, and adjuvant treatment had the best overall survival. Our data supports recent studies that have shown perioperative chemotherapy and/or adjuvant treatment significantly improves overall survival in patients with resectable gastric cancer[39,43,44]. Because no clinical trials addressing the benefits of perioperative and adjuvant treatment included AN patients, our study suggest AN patients also benefit from having perioperative and/or adjuvant treatment with surgery. However, a randomized clinical trial that included AN patients would be necessary to confirm this finding. In addition to surgery, patients who had more than 15 regional lymph nodes examined at the time of resection

had a better overall survival when compared to patients who had less than 15 lymph nodes examined. Previous studies have shown lower recurrence and increased survival in patients who received extended lymph node dissection with a D2 lymphadenectomy of 15 or more lymph nodes[45,46]. However, these results have not been consistent across all studies[47,48], and this issue remains controversial.

There are limitations to our study. First, the small number of AN gastric cancer cases diagnosed at ANMC from 2006-2014 limited our power to detect modest associations. The AN population is relatively small-consisting of 150000 people. In order to conduct this study, we reviewed all AN gastric cancer cases diagnosed at the ANMC between 2006-2014, approximately 132 cases. Records from this timespan had the epidemiological information needed to conduct this study, which is why we focused on these individuals. Even with the small number of cases, we were able to detect significant differences in the results. Although the AN population is small, we feel this population is worthy of study because of the poor clinical outcomes and gastric cancer mortality rates that are unique to this population within Alaska. Second, as a retrospectively assembled surveillance study, some relevant confounders were not documented and could not be assessed; for example, blood type has been shown to correlate with gastric cancer prognosis, but was not assessed in 20% of our patients. Future studies need to include relevant confounders, particularly blood type. In addition, family history and tobacco use are captured in AN medical records as patient-reported, which may result in artificially lower percentages due to under reporting[49,50]. Finally, our retrospective surveillance study of AN gastric patients was selected from the ANMC hospital-based registry, which represents approximately 80% of AN gastric cancer cases reported to the SEER-funded AN Tumor Registry. The 20% of patients not in the hospital registry are most likely living outside of Alaska, receiving care at private hospitals, or traveling to other regions of the US for treatment. For accuracy, it would be beneficial to have data on all AN patients, but due to our limitations, the current study uses only patients who were cared for in Alaska at ANMC. It is possible that by not including all AN people we are introducing an element of selection bias into our results, however similar trends in clinical or pathological characteristics were observed between the AN SEER and AN ANMC Hospital-based registries. By utilizing the ANMC hospital-based registry we were able to further evaluate clinicopathological and treatment outcomes that were not collected by the SEER registry.

In summary, gastric cancer in AN people is distinct from the NHW population. AN patients were observed to have increased incidence, poorer prognosis, earlier age of diagnosis, and variation in location, and subtype of gastric cancer. These clinicopathological characteristics could be driven by multiple variables including, socioeconomic

factors and biological differences, such as lifestyle differences, genetic alterations, and environmental exposures. Our findings confirm the importance of early detection, treatment, and surgical resection for AN patients with resectable gastric adenocarcinoma in order to optimize patient outcomes. This study highlights the need for further investigation into understanding the basis for the increased incidence and poorer prognosis of this devastating cancer in AN people.

ARTICLE HIGHLIGHTS

Research background
Gastric cancer is a leading cancer health disparity among the Alaska Native (AN) people, with a 3-fold higher incidence and mortality rate compared to United States non-Hispanic White (NHW) people. There are currently a paucity of studies investigating the clinicopathologic features of this disease in AN people, and their relationship to clinical outcomes.

Research motivation
This study was conducted to gain a deeper understanding of AN gastric cancer patient characteristics, pathologic variables, clinical patterns of care, and patient outcomes to gain insights into to this cancer health disparity.

Research objectives
In order to further investigate how to reduce gastric cancer incidence and mortality rates among the AN population, we sought to evaluate recent trends in gastric cancer incidence, response to treatment, and overall survival outcomes in this high incidence population. A greater understanding of gastric cancer incidence and response to treatment among the AN people may facilitate the design of screening programs or the identification of early detection measures, and elucidate new areas for future investigation to potentially reduce incidence and improve patient outcomes.

Research methods
We performed a retrospective analysis of 132 AN gastric cancer patients treated at the Alaska Native Medical Center (ANMC) from 2006-2014, utilizing the ANMC Tumor Registry and manual patient chart reviews. We compared our findings to data on United States (US) NHW and AN gastric adenocarcinoma patients obtained from the US National Institute's SEER Program of the National Cancer Institute 18 dataset for the period 2006-2014. Data were analyzed using software SPSS 23.0.

Research results
AN patients differ from NHW patients in that they have a higher prevalence of non-cardia tumors, unique histological features with a higher incidence of the diffuse subtype, and a higher incidence of signet ring cell carcinomas. AN females were more likely to be diagnosed with stage IV cancers compared to AN males. We observed a decreased overall survival among AN patients with advanced stage disease, O+ blood type, < 15 lymph nodes examined at resection, and no treatment. AN gastric cancer patients have a higher incidence rate, a poorer overall survival, and are diagnosed at a significantly younger age compared to NHW patients. This study is the first report detailing the clinicopathologic features of gastric cancer in AN people, as well as information on patterns of care, and clinical outcome data.

Research conclusions
Gastric cancer in AN people is distinct from the NHW population. AN patients were observed to have increased incidence, poorer prognosis, earlier age of diagnosis, and variation in location, and histological subtype of gastric cancer. These clinicopathological characteristics could be driven by multiple variables including, socioeconomic factors and biological differences, such as lifestyle differences, genetic alterations, and environmental exposures. Our findings confirm the importance of early detection, treatment, and surgical resection for AN patients with resectable gastric adenocarcinoma in order to optimize

patient outcomes. This study highlights the need for further investigation into understanding the basis for the increased incidence and poorer prognosis of this devastating cancer in AN people.

Research perspectives

Our work highlights the unique clinical and pathologic features of gastric cancer in the AN population. The high incidence of this cancer warrants prompt referral for endoscopic evaluation of AN patients presenting with gastrointestinal symptoms. Of particular concern is the finding that younger women present more frequently with stage IV disease, emphasizing the need to consider a diagnosis of gastric cancer earlier in this population. Clinical outcomes are poor in this population, despite the fact that patients are treated according to standard guidelines. An important area for future study will be investigations into the molecular features of gastric cancer in AN people, with the goal of identifying new prognostic and predictive markers that may improve treatment regimens, and possibly identify new targets for precision medicine.

REFERENCES

1   **Ferlay J**, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015; **136**: E359-E386 [PMID: 25220842 DOI: 10.1002/ijc.29210]

2   **Sierra MS**, Cueva P, Bravo LE, Forman D. Stomach cancer burden in Central and South America. *Cancer Epidemiol* 2016; **44** Suppl 1: S62-S73 [PMID: 27678324 DOI: 10.1016/j.canep.2016.03.008]

3   **Rahman R**, Asombang AW, Ibdah JA. Characteristics of gastric cancer in Asia. *World J Gastroenterol* 2014; **20**: 4483-4490 [PMID: 24782601 DOI: 10.3748/wjg.v20.i16.4483]

4   **Crew KD**, Neugut AI. Epidemiology of gastric cancer. *World J Gastroenterol* 2006; **12**: 354-362 [PMID: 16489633 DOI: 10.3748/wjg.v12.i3.354]

5   **Lee E**, Liu L, Zhang J, Stern MC, Barzi A, Hwang A, Kim AE, Hamilton AS, Wu AH, Deapen D. Stomach Cancer Disparity among Korean Americans by Tumor Characteristics: Comparison with Non-Hispanic Whites, Japanese Americans, South Koreans, and Japanese. *Cancer Epidemiol Biomarkers Prev* 2017; **26**: 587-596 [PMID: 27908922 DOI: 10.1158/1055-9965.EPI-16-0573]

6   **Carmack AM**, Schade TL, Sallison I, Provost EM, Kelly JJ. Cancer in Alaska Native People: 1969-2013: The 45 Year Report. Anchorage, AK: Alaska Native Tumor Registry, Alaska Native Epidemiology Center, Alaska Native Tribal Health Consortium, 2015

7   **Arnold M**, Moore SP, Hassler S, Ellison-Loschmann L, Forman D, Bray F. The burden of stomach cancer in indigenous populations: a systematic review and global assessment. *Gut* 2014; **63**: 64-71 [PMID: 24153248 DOI: 10.1136/gutjnl-2013-305033]

8   **Alberts SR**, Kelly JJ, Lanier AP, Sacco F. Occurrence of esophageal and gastric cancer in Alaska Natives, 1969-2003. *Alaska Med* 2006; **48**: 2-11 [PMID: 17042389]

9   **Wiggins CL**, Perdue DG, Henderson JA, Bruce MG, Lanier AP, Kelley JJ, Seals BF, Espey DK. Gastric cancer among American Indians and Alaska Natives in the United States, 1999-2004. *Cancer* 2008; **113**: 1225-1233 [PMID: 18720378 DOI: 10.1002/cncr.23732]

10 **Lanier AP**, Kelly JJ, Maxwell J, McEvoy T, Homan C. Cancer in Alaska Natives 1969-2003 35-year Report. Alaska Native Tribial Health Consortium. Anchorage, AK, 2006

11 **Jemal A**, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90 [PMID: 21296855 DOI: 10.3322/caac.20107]

12 **Devesa SS**, Blot WJ, Fraumeni JF Jr. Changing patterns in the incidence of esophageal and gastric carcinoma in the United States. *Cancer* 1998; **83**: 2049-2053 [PMID: 9827707]

13 **Camargo MC**, Murphy G, Koriyama C, Pfeiffer RM, Kim WH, Herrera-Goepfert R, Corvalan AH, Carrascal E, Abdirad A, Anwar M, Hao Z, Kattoor J, Yoshiwara-Wakabayashi E, Eizuru Y, Rabkin CS, Akiba S. Determinants of Epstein-Barr virus-positive gastric cancer: an international pooled analysis. *Br J Cancer* 2011; **105**: 38-43 [PMID: 21654677 DOI: 10.1038/bjc.2011.215]

14 **Keck JW**, Miernyk KM, Bulkow LR, Kelly JJ, McMahon BJ, Sacco F, Hennessy TW, Bruce MG. Helicobacter pylori infection and markers of gastric cancer risk in Alaska Native persons: a retrospective case-control study. *Can J Gastroenterol Hepatol* 2014; **28**: 305-310 [PMID: 24945184]

15 **Sacco F**, Bruce MG, McMahon BJ, Bruden D. A prospective evaluation of 200 upper endoscopies performed in Alaska Native persons. *Int J Circumpolar Health* 2007; **66**: 144-152 [PMID: 17515254]

16 **Lauren P**. The Two Histological Main Types of Gastric Carcinoma: Diffuse and So-Called Intestinal-Type Carcinoma. An Attempt at a Histo-Clinical Classification. *Acta Pathol Microbiol Scand* 1965; **64**: 31-49 [PMID: 14320675]

17 **Washington K**. 7th edition of the AJCC cancer staging manual: stomach. *Ann Surg Oncol* 2010; **17**: 3077-3079 [PMID: 20882416 DOI: 10.1245/s10434-010-1362-z]

18 **Ajani JA**, Bentrem DJ, Besh S, D'Amico TA, Das P, Denlinger C, Fakih MG, Fuchs CS, Gerdes H, Glasgow RE, Hayman JA, Hofstetter WL, Ilson DH, Keswani RN, Kleinberg LR, Korn WM, Lockhart AC, Meredith K, Mulcahy MF, Orringer MB, Posey JA, Sasson AR, Scott WJ, Strong VE, Varghese TK Jr, Warren G, Washington MK, Willett C, Wright CD, McMillian NR, Sundar H; National Comprehensive Cancer Network. Gastric cancer, version 2.2013: featured updates to the NCCN Guidelines. *J Natl Compr Canc Netw* 2013; **11**: 531-546 [PMID: 23667204]

19 **Dhobi MA**, Wani KA, Parray FQ, Wani RA, Wani ML, Peer GQ, Abdullah S, Wani IA, Wani MA, Shah MA, Thakur N. Gastric cancer in young patients. *Int J Surg Oncol* 2013; **2013**: 981654 [PMID: 24381753 DOI: 10.1155/2013/981654]

20 **Theuer CP**, de Virgilio C, Keese G, French S, Arnell T, Tolmos J, Klein S, Powers W, Oh T, Stabile BE. Gastric adenocarcinoma in patients 40 years of age or younger. *Am J Surg* 1996; **172**: 473-476; discussion 476-477 [PMID: 8942547 DOI: 10.1016/S0002-9610(96)00223-1]

21 **Anderson WF**, Rabkin CS, Turner N, Fraumeni JF Jr, Rosenberg PS, Camargo MC. The Changing Face of Noncardia Gastric Cancer Incidence Among US Non-Hispanic Whites. *J Natl Cancer Inst* 2018; **110**: 608-615 [PMID: 29361173 DOI: 10.1093/jnci/djx262]

22 **Taghavi S**, Jayarajan SN, Davey A, Willis AI. Prognostic significance of signet ring gastric cancer. *J Clin Oncol* 2012; **30**: 3493-3498 [PMID: 22927530 DOI: 10.1200/JCO.2012.42.6635]

23 **Liu X**, Cai H, Sheng W, Yu L, Long Z, Shi Y, Wang Y. Clinicopathological Characteristics and Survival Outcomes of Primary Signet Ring Cell Carcinoma in the Stomach: Retrospective Analysis of Single Center Database. *PLoS One* 2015; **10**: e0144420 [PMID: 26642199 DOI: 10.1371/journal.pone.0144420]

24 **Parkinson AJ**, Gold BD, Bulkow L, Wainwright RB, Swaminathan B, Khanna B, Petersen KM, Fitzgerald MA. High prevalence of Helicobacter pylori in the Alaska native population and association with low serum ferritin levels in young adults. *Clin Diagn Lab Immunol* 2000; **7**: 885-888 [PMID: 11063492]

25 **Corral JE**, Delgado Hurtado JJ, Domínguez RL, Valdez de Cuéllar M, Balmore Cruz C, Morgan DR. The descriptive epidemiology of gastric cancer in Central America and comparison with United States Hispanic populations. *J Gastrointest Cancer* 2015; **46**: 21-28 [PMID: 25412859 DOI: 10.1007/s12029-014-9672-1]

26 **Fock KM**, Ang TL. Epidemiology of Helicobacter pylori infection and gastric cancer in Asia. *J Gastroenterol Hepatol* 2010; **25**: 479-486 [PMID: 20370726 DOI: 10.1111/j.1440-1746.2009.06188.x]

27 **Kang SY**, Han JH, Ahn MS, Lee HW, Jeong SH, Park JS, Cho

YK, Han SU, Kim YB, Kim JH, Sheen SS, Lim HY, Choi JH. Helicobacter pylori infection as an independent prognostic factor for locally advanced gastric cancer patients treated with adjuvant chemotherapy after curative resection. *Int J Cancer* 2012; **130**: 948-958 [PMID: 21425257 DOI: 10.1002/ijc.26081]

28 **Tveit AH**, Bruce MG, Bruden DL, Morris J, Reasonover A, Hurlburt DA, Hennessy TW, McMahon B. Alaska sentinel surveillance study of Helicobacter pylori isolates from Alaska Native persons from 2000 to 2008. *J Clin Microbiol* 2011; **49**: 3638-3643 [PMID: 21813726 DOI: 10.1128/JCM.01067-11]

29 **Miernyk K**, Morris J, Bruden D, McMahon B, Hurlburt D, Sacco F, Parkinson A, Hennessy T, Bruce M. Characterization of Helicobacter pylori cagA and vacA genotypes among Alaskans and their correlation with clinical disease. *J Clin Microbiol* 2011; **49**: 3114-3121 [PMID: 21752979 DOI: 10.1128/JCM.00469-11]

30 **Blaser MJ**, Perez-Perez GI, Kleanthous H, Cover TL, Peek RM, Chyou PH, Stemmermann GN, Nomura A. Infection with Helicobacter pylori strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer Res* 1995; **55**: 2111-2115 [PMID: 7743510]

31 **Parsonnet J**, Friedman GD, Orentreich N, Vogelman H. Risk for gastric cancer in people with CagA positive or CagA negative Helicobacter pylori infection. *Gut* 1997; **40**: 297-301 [PMID: 9135515]

32 **Van Doorn LJ**, Figueiredo C, Mégraud F, Pena S, Midolo P, Queiroz DM, Carneiro F, Vanderborght B, Pegado MD, Sanna R, De Boer W, Schneeberger PM, Correa P, Ng EK, Atherton J, Blaser MJ, Quint WG. Geographic distribution of vacA allelic types of Helicobacter pylori. *Gastroenterology* 1999; **116**: 823-830 [PMID: 10092304]

33 **Karimi P**, Islami F, Anandasabapathy S, Freedman ND, Kamangar F. Gastric cancer: descriptive epidemiology, risk factors, screening, and prevention. *Cancer Epidemiol Biomarkers Prev* 2014; **23**: 700-713 [PMID: 24618998 DOI: 10.1158/1055-9965.EPI-13-1057]

34 **Kelley JJ**, Schade TL, Starkey BM, White S, Ashokkumar R, Lanier AP. Cancer in Alaska Native People 1969-2008 a 40-Year Report. Alaska Native Tribial Health Consortium. Anchorage, AK, 2012

35 **Lanier AP**, Alberts SR. Cancers of the buccal cavity and pharynx in Circumpolar Inuit. *Acta Oncol* 1996; **35**: 545-552 [PMID: 8813061]

36 **Walker B**, Davidson V, Butler J, Allely K. Alaska Tobacco Facts 2015 Update. 2015

37 **Fox JG**, Wang TC. Inflammation, atrophy, and gastric cancer. *J Clin Invest* 2007; **117**: 60-69 [PMID: 17200707 DOI: 10.1172/JCI30111]

38 **Yamaoka Y**, Kato M, Asaka M. Geographic differences in gastric cancer incidence can be explained by differences between Helicobacter pylori strains. *Intern Med* 2008; **47**: 1077-1083 [PMID: 18552463]

39 **GASTRIC (Global Advanced/Adjuvant Stomach Tumor Research International Collaboration) Group**, Paoletti X, Oba K, Burzykowski T, Michiels S, Ohashi Y, Pignon JP, Rougier P, Sakamoto J, Sargent D, Sasako M, Van Cutsem E, Buyse M. Benefit of adjuvant chemotherapy for resectable gastric cancer: a meta-analysis. *JAMA* 2010; **303**: 1729-1737 [PMID: 20442389 DOI: 10.1001/jama.2010.534]

40 **Edgren G**, Hjalgrim H, Rostgaard K, Norda R, Wikman A, Melbye M, Nyrén O. Risk of gastric cancer and peptic ulcers in relation to ABO blood type: a cohort study. *Am J Epidemiol* 2010; **172**: 1280-1285 [PMID: 20937632 DOI: 10.1093/aje/kwq299]

41 **Wang Z**, Liu L, Ji J, Zhang J, Yan M, Zhang J, Liu B, Zhu Z, Yu Y. ABO blood group system and gastric cancer: a case-control study and meta-analysis. *Int J Mol Sci* 2012; **13**: 13308-13321 [PMID: 23202954 DOI: 10.3390/ijms131013308]

42 **Qiu MZ**, Zhang DS, Ruan DY, Luo HY, Wang ZQ, Zhou ZW, Wang FH, Li YH, Xu RH. A relationship between ABO blood groups and clinicopathologic characteristics of patients with gastric adenocarcinoma in China. *Med Oncol* 2011; **28** Suppl 1: S268-S273 [PMID: 21053099 DOI: 10.1007/s12032-010-9735-5]

43 **Knight G**, Earle CC, Cosby R, Coburn N, Youssef Y, Malthaner R, Wong RK; Gastrointestinal Cancer Disease Site Group. Neoadjuvant or adjuvant therapy for resectable gastric cancer: a systematic review and practice guideline for North America. *Gastric Cancer* 2013; **16**: 28-40 [PMID: 22467061 DOI: 10.1007/s10120-012-0148-3]

44 **Ikoma N**, Cormier JN, Feig B, Du XL, Yamal JM, Hofstetter W, Das P, Ajani JA, Roland CL, Fournier K, Royal R, Mansfield P, Badgwell BD. Racial disparities in preoperative chemotherapy use in gastric cancer patients in the United States: Analysis of the National Cancer Data Base, 2006-2014. *Cancer* 2018; **124**: 998-1007 [PMID: 29393964 DOI: 10.1002/cncr.31155]

45 **Biondi A**, D'Ugo D, Cananzi FC, Papa V, Borasi A, Sicoli F, Degiuli M, Doglietto G, Persiani R. Does a minimum number of 16 retrieved nodes affect survival in curatively resected gastric cancer? *Eur J Surg Oncol* 2015; **41**: 779-786 [PMID: 25899981 DOI: 10.1016/j.ejso.2015.03.227]

46 **Datta J**, Lewis RS Jr, Mamtani R, Stripp D, Kelz RR, Drebin JA, Fraker DL, Karakousis GC, Roses RE. Implications of inadequate lymph node staging in resectable gastric cancer: a contemporary analysis using the National Cancer Data Base. *Cancer* 2014; **120**: 2855-2865 [PMID: 24854027 DOI: 10.1002/cncr.28780]

47 **Hartgrink HH**, van de Velde CJ, Putter H, Bonenkamp JJ, Klein Kranenbarg E, Songun I, Welvaart K, van Krieken JH, Meijer S, Plukker JT, van Elk PJ, Obertop H, Gouma DJ, van Lanschot JJ, Taat CW, de Graaf PW, von Meyenfeldt MF, Tilanus H, Sasako M. Extended lymph node dissection for gastric cancer: who may benefit? Final results of the randomized Dutch gastric cancer group trial. *J Clin Oncol* 2004; **22**: 2069-2077 [PMID: 15082726 DOI: 10.1200/JCO.2004.08.026]

48 **El-Sedfy A**, Dixon M, Seevaratnam R, Bocicariu A, Cardoso R, Mahar A, Kiss A, Helyer L, Law C, Coburn NG. Personalized Surgery for Gastric Adenocarcinoma: A Meta-analysis of D1 versus D2 Lymphadenectomy. *Ann Surg Oncol* 2015; **22**: 1820-1827 [PMID: 25348779 DOI: 10.1245/s10434-014-4168-6]

49 **Murff HJ**, Spigel DR, Syngal S. Does this patient have a family history of cancer? An evidence-based analysis of the accuracy of family cancer history. *JAMA* 2004; **292**: 1480-1489 [PMID: 15383520 DOI: 10.1001/jama.292.12.1480]

50 **Connor Gorber S**, Schofield-Hurwitz S, Hardt J, Levasseur G, Tremblay M. The accuracy of self-reported smoking: a systematic review of the relationship between self-reported and cotinine-assessed smoking status. *Nicotine Tob Res* 2009; **11**: 12-24 [PMID: 19246437 DOI: 10.1093/ntr/ntn010]

Observational Study

Transforming growth factor-β and peripheral regulatory cells are negatively correlated with the overall survival of hepatocellular carcinoma

Yang An, Song Gao, Wen-Chao Zhao, Bao-An Qiu, Nian-Xin Xia, Peng-Jun Zhang, Zhen-Ping Fan

Yang An, Wen-Chao Zhao, Bao-An Qiu, Nian-Xin Xia, Department of Hepato-Biliary-Pancreatic Surgery, Navy General Hospital of Chinese People's Liberation Army, Beijing 100048, China

Song Gao, Peng-Jun Zhang, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Department of Interventional Therapy, Peking University Cancer Hospital & Institute, Beijing 100142, China

Zhen-Ping Fan, Liver Disease Center for Cadre Medical Care, Beijing 302 Military Hospital, Beijing 100039, China

ORCID number: Yang An (0000-0002-2515-4562); Song Gao (0000-0002-7466-3654); Wen-Chao Zhao (0000-0002-1445-376X); Bao-An Qiu (0000-0001-9474-3101); Nian-Xin Xia (0000-0002-3785-1381); Peng-Jun Zhang (0000-0002-7391-2495); Zhen-Ping Fan (0000-0001-5725-5758).

Institutional review board statement: The study was reviewed and approved by the Peking University Cancer Hospital & Institute review board.

Informed consent statement: All study participants or their legal guardian provided written informed consent prior to study enrollment.

Conflict-of-interest statement: We declare that we have no financial or personal relationships with other individuals or organizations that can inappropriately influence our work and that there is no professional or other personal interest of any nature in any product, service, and/or company that could be construed as influencing the position presented in or the review of the manuscript.

Data sharing statement: No additional data are available.

STROBE statement: The guidelines of the STROBE Statement have been adopted in our manuscript.

Abstract

AIM

To understand the cellular and molecular changes in

peripheral blood that can lead to the development of hepatocellular carcinoma (HCC) and provide new methods for its diagnosis and treatment.

METHODS
Peripheral blood mononuclear cells were isolated from the peripheral blood of HCC patients and normal controls and then analyzed by flow cytometry. The percentage of transforming growth factor-β (TGF-β)+ regulatory cells (Tregs) in the peripheral blood was measured, and the expression of TGF-β was also determined. Then, the relationship between the changes and the 5-year survival of patients was analyzed. In addition, recombinant human TGF-β (rhTGF-β) and recombinant human interleukin-6 were added to stimulate the cultured cells, and their effects on HCC were evaluated.

RESULTS
The expression of TGF-β and the percentage of TGF-β+ Tregs in the peripheral blood of HCC patients increased significantly compared with normal controls. Compared with the low TGF-β expression group, the high TGF-β expression group had a significantly lower 5-year survival rate, and the same result was found in the two TGF-β+ Treg groups, suggesting that TGF-β and TGF-β+ Tregs were negatively correlated with the overall survival of the patients. In addition, rhTGF-β promoted the growth of tumor cells and induced high expression levels of IL-6, which further promoted tumor proliferation.

CONCLUSION
The results showed that TGF-β may promote tumor growth and proliferation by inducing the production of IL-6, and TGF-β and TGF-β+ Tregs may serve as new markers for predicting a poor prognosis in HCC.

Key words: Hepatocellular carcinoma; Transforming growth factor-β; Regulatory cells; Peripheral blood mononuclear cells; Interleukin-6

Core tip: We aimed to understand the cellular and molecular changes in peripheral blood of hepatocellular carcinoma (HCC) and provide new methods for its diagnosis and treatment. The results showed that transforming growth factor-β (TGF-β) may promote tumor growth and proliferation by inducing the production of interleukin-6, and TGF-β and TGF-β+ regulatory cells may serve as new markers for predicting poor prognosis of HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the second leading cause of cancer deaths in the word[1]. According to the World Health Organization (WHO), approximately 788000 people die from primary liver cancer each year. It has been reported that the morbidity and mortality of HCC in China are very high[2,3]. Improvements have been made in HCC treatment, including surgery, chemotherapy, radiation therapy, and ablation; however, HCC still has a poor prognosis because of recurrence and tumor metastasis[4].

Studies have shown that tumors can establish the tumor microenvironment by regulating lymphocytes, which play an important role in tumor development through inflammatory immunity[5,6]. Tumor cells can inhibit the function of tumor infiltrating lymphocytes (TILs) in the tumor microenvironment through inhibitory signaling pathways in the immune system; this process results in the immunosuppression of tumors[7,8]. Tumors can also accumulate infiltrated immune cells to inhibit anti-tumor effects by secreting immunosuppressive factors, such as interleukin-2 (IL-2), transforming growth factor-β (TGF-β), matrix metalloproteinase (MMP), vascular endothelial growth factor (VEGF), and interleukin-10 (IL-10), into the microenvironment[9]. It has also been reported that tumors can affect the proliferation, migration, and survival of cancer cells through the inflammatory effects of immune cells[10,11]. It is well known that regulatory cells (Tregs) can promote the development of tumors by inhibiting immune surveillance[12]. The knockdown of forkhead box P3 (Foxp3), which is specifically expressed in Tregs, can reduce the immunosuppression of Tregs, which can suppress tumor growth in mice with HCC[13]. Therefore, understanding the immune status, especially the inflammatory state of cancer, may provide a new method of immunomodulation for the treatment of cancer.

Studies have shown that TGF-β is one of the key products of Tregs[14], and it maintains the internal stability of stem cells and promotes fibrosis, embryonic development, tissue repair, cell proliferation and differentiation, and immune regulation[15]. Tumor-associated TGF-β negatively regulates the host immune response through the following mechanisms[16,17]. First, tumor-associated TGF-β mediates the differentiation of Th1 cells to Th2 cells *via* IL-10. Second, tumor-associated TGF-β directly inhibits M1 giant macrophages and the antitumor immune responses mediated by Th1. Third, tumor-associated TGF-β inhibits the functions of CD8+ T lymphocytes, natural killer (NK) cells, and dendritic cells, which have cytotoxic effects. Fourth, tumor-associated TGF-β produces CD4+ CD25+ Tregs to inhibit the function of other lymphocyte groups[18]. Finally, tumor-associated TGF-β promotes M2 macrophages, which can produce reactive oxygen species to promote tumor development through secreting immunosuppressive cytokines (TGF-β and IL-10), angiogenic factors (CXC chemokines, MMP-9, and VEGF), proinflammatory

Table 1 Clinicopathological characteristics of 100 patients with hepatocellular carcinoma *n* (%)			
Variables	**HCC (*n* = 100)**	**Normal controls (*n* = 36)**	***P* value**
Average age (yr)	53.33	50.36	0.16
Age (yr)			0.61
≤ 50	45 (45.0)	18 (50.0)	
> 50	55 (55.0)	18 (50.0)	
Sex			0.68
Male	65 (65.0)	22 (61.1)	
Female	35 (35.0)	14 (38.9)	
T classification			
T1	12 (12.0)		
T2	36 (36.0)		
T3	42 (42.0)		
T4	10 (10.0)		

HCC: Hepatocellular carcinoma.

cytokines (IL-1, TNF-α, and IL-6) and tumor growth factors. TGF-β has a dual role in the development of tumors, and it can promote the invasion and metastasis of tumor cells mainly by regulating the immune system and tumor microenvironment[16].

MATERIALS AND METHODS

Patients and blood sample collection
We included 100 patients with HCC who were diagnosed at Beijing Cancer Hospital between 2010 and 2014, and we included 36 healthy subjects without any signs of disease. Blood samples were collected before any treatment. According to the 2002 American Joint Committee on Cancer (AJCC) staging criteria, the samples were divided into two groups: Early stage (stages I and II) and late stage (stages III and IV). All patient information is shown in Table 1. Of the HCC patients, 63 were followed up until death or until July 31, 2017. The median follow-up time was 40.3 mo (mean ± SD: 40.3 ± 11.6 mo). All participants provided informed consent, and the study was approved by the Ethics Committee of Beijing Cancer Hospital.

Measurement of cytokines
Whole blood samples from all patients and normal controls were centrifuged at 3000 g for 10 min to collect the serum; then, the concentrations of TGF-β and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, Waltham, MA, United States) and human inflammation CBA kits (BD Biosciences, San Jose, CA, United States), respectively.

Collection and culture of peripheral blood mononuclear cells (PBMCs)
Density centrifugation of 5 mL of heparinized peripheral blood from patients with HCC and normal controls was performed to obtain PBMCs. There were two steps of the horizontal centrifugation: 2000 r/min for 20 min and 15000 r/min for 10 min[19]. The final cell pellet was resuspended and cultured in the appropriate amount of 1640 medium containing 10% fetal bovine serum.

The cells were counted using a microscope, and the cell concentration was adjusted to 2×10^6/mL. A total of 200 μL of each cell suspension was pipetted into a 96-well flat bottom cell culture plate, and 1 μL of 10 μg/mL PMA, 2 μL of 100 μg/mL IO, and 2 μL of 300 μg/mL BFA were added. The cells were incubated at 37 ℃ with 5% CO_2 for 4-5 h and then subjected to flow cytometry and analysis.

Flow cytometry analysis
CD4 and CD3 antibodies were added to label the cells. A Foxp3 antibody was also added to detect the Tregs. A constant volume of 100 μL of phosphate buffered saline (PBS) was used for the flow cytometry analyses, which were performed on a BD FACSCalibur system.

Cell culture
HepG2, PLHC-1, and LMH cell lines were selected for cell culture, and all three cell lines were cultured in an incubator at 37 ℃ and 5% CO_2. rhTGF-β or rhIL-6 (PeproTech, Rocky Hill, NJ, United States) was added to three 6-well plates containing the three HCC cell lines to stimulate the tumor cells for 24-48 h according to the manufacturer's instructions. To analyze the proliferation rates, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays and cell counting were performed.

Statistical analysis
Statistical analyses were performed using GraphPad Prism 5.0 (San Diego, CA, United States). The data are expressed as the mean ± SD. T-tests were used to assess the differences between groups. The overall survival rates of HCC were analyzed with Kaplan-Meier curves. All tests were two-tailed, and $P < 0.05$ was considered to be statistically significant.

RESULTS

Increased expression of TGF-β in the serum of liver cancer patients
We measured the levels of TGF-β in the serum of 100
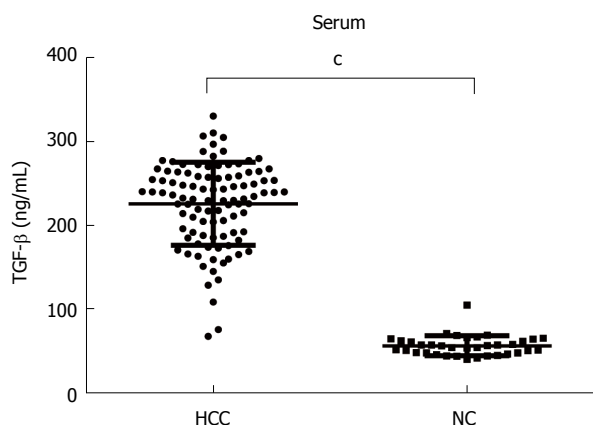
Serum

Figure 1 Levels of transforming growth factor-β in the serum of hepatocellular carcinoma patients and normal controls. $^{c}P < 0.001$. TGF-β: Transforming growth factor-β; HCC: Hepatocellular carcinoma.

patients with HCC and 36 normal controls using ELISAs. The results showed that the serum levels of TGF-β were significantly higher in HCC patients (225.82 ± 48.93 ng/mL) than in normal controls (57.29 ± 11.70 ng/mL, $P < 0.0001$, Figure 1).

Tregs are associated with the progression of HCC

Studies have shown that TGF-β is one of the key products of Tregs[14]. We performed flow cytometry analyses of 100 HCC samples and 36 normal controls, and the results clearly indicated TGF-β-expressing cell populations in the PBMCs and Tregs. The population of TGF-β-expressing cells in the Tregs was analyzed by flow cytometry, and Figure 2A is representative of all the data figures. A statistical analysis showed that the proportion of TGF-β+ Tregs was significantly higher in HCC patients (14.14 ± 6.02%) than in normal controls (3.00 ± 1.43%, $P <0.001$, Figure 2B). In fact, the percentage of TGF-β+ Tregs was significantly higher in HCC patients in advanced stages (15.47 ± 6.95%) than in HCC patients in early stages (12.70 ± 4.37%) and normal controls (Figure 2C), suggesting a possible positive correlation between the presence of TGF-β+ Tregs and tumor progression.

Increased levels of TGF-β and TGF-β+ Tregs may predict a poor prognosis for HCC

In this study, we analyzed the effects of high and low levels of peripheral blood TGF-β and TGF-β+ Tregs on the five-year survival rate of 63 patients with HCC who were followed up. The results showed that compared to the low TGF production group, the high TGF-β production group had a significantly lower overall survival rate (Figure 3A, 31.0% *vs* 53.0%, $P = 0.010$). Compared with the low TGF-β+ Tregs group, the high TGF-β+ Tregs group had a significantly higher death hazard rate (Figure 3B, 38.0% *vs* 53.5%, $P = 0.047$). The results suggest that the overall survival of the patients with HCC is negatively correlated with the

levels of TGF and Tregs.

TGF-β activates IL-6 production in HCC to promote tumor growth

Three human HCC cell lines, namely, HepG2, PLHC-1, and LMH, were selected for this study. We added rhTGF-β to stimulate the three HCC cell lines and then detected changes in their biological function. Compared with the untreated group, all three HCC cell lines treated with 100 ng/mL rhTGF-β for 48 h showed significant cell proliferation afterwards (Figure 4A). We also examined IL-6 levels in the cell supernatants and found that IL-6 levels were significantly higher in the rhTGF-β-treated cell supernatants than in the untreated cell supernatants (Figure 4B). Furthermore, the three HCC cell lines were treated with rhIL-6, and significantly higher cell proliferation rates were found in all three HCC cell lines treated with rhIL-6 than in the untreated cells (Figure 4C). These results indicate that TGF-β has the potential to promote HCC cell proliferation, and it can also mediate IL-6 production to promote further tumor cell proliferation.

DISCUSSION

This study demonstrated that the levels of TGF-β and TGF-β+ Tregs in the peripheral blood were significantly higher in HCC patients than in normal controls, and these levels were negatively associated with the 5-year survival of HCC patients. At the same time, TGF may promote the development of tumors by inducing IL-16 expression, suggesting that peripheral TGF-β and TGF-β+ Tregs can be used as potential markers to predict a poor prognosis in HCC patients.

Understanding the immune status, especially the inflammatory state of cancer, may provide a new method of immunomodulation for the treatment of cancer. In the early stages of cancer, or the stages of hepatocellular degeneration and injury, abnormally high expression levels of IGF-II have been observed, which can be an indicator for the early diagnosis of liver cancer. Liver injury may be caused by the abnormal activation of the IGF-II gene by the cancer-causing factor diethylnitrosamine[20,21]. It was also reported that high expression levels of IGF-II in the serum and livers of rats are events in the early and advanced stages of the occurrence and development of liver cancer[21]. Some studies have shown that VEGF is more sensitive for the prediction of liver cancer in patients with cirrhosis related to hepatitis C virus (HCV), and VEGF expression is related to tumor vascular invasion[22]. VEGF expression has been associated with tumor diameter and lymph node metastasis, and its positive rate is 72.0% in HCC. Therefore, VEGF may be applied for evaluating hematopoietic liver cancer metastasis and predicting HCC with HCV. It has been reported that the expression levels of Golgi protein 73 (GP73) are significantly higher in HCC serum samples than
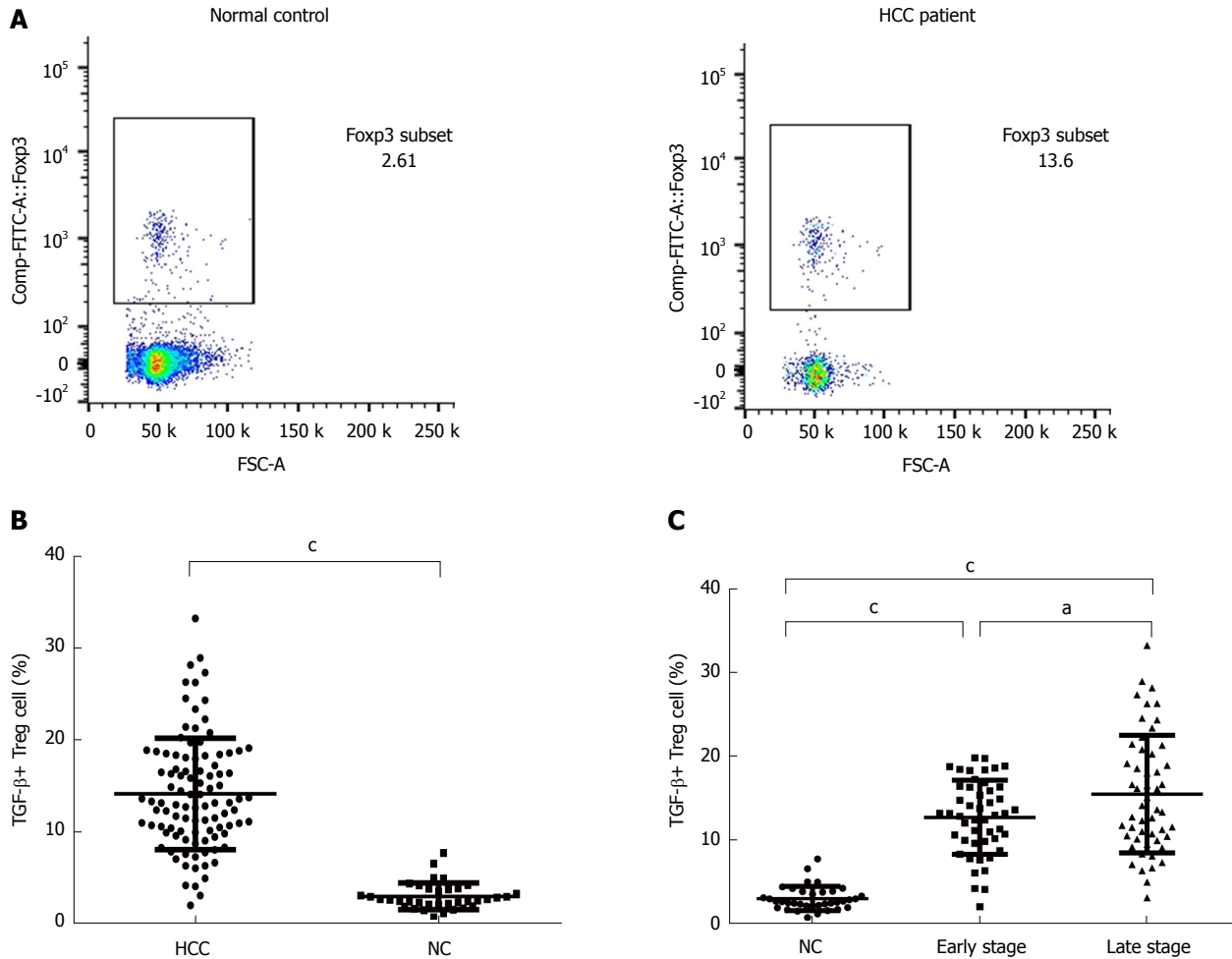
Figure 2 Increase of transforming growth factor-β+ regulatory cells in the serum of hepatocellular carcinoma patients. A: Representative plots for identification of the TGF-β+ Treg cells producing cells in PBMC; B: Analysis of the frequency of TGF-β+ Treg cell from the PBMCs of healthy donors in comparison to HCC patients; C: Analysis of the frequency of TGF-β+ Treg cell from the PBMCs of healthy donors in comparison to HCC patients with tumors at different stages. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. TGF-β: Transforming growth factor-β; Treg: Regulatory cells; PBMC: Peripheral blood mononuclear cells; HCC: Hepatocellular carcinoma.
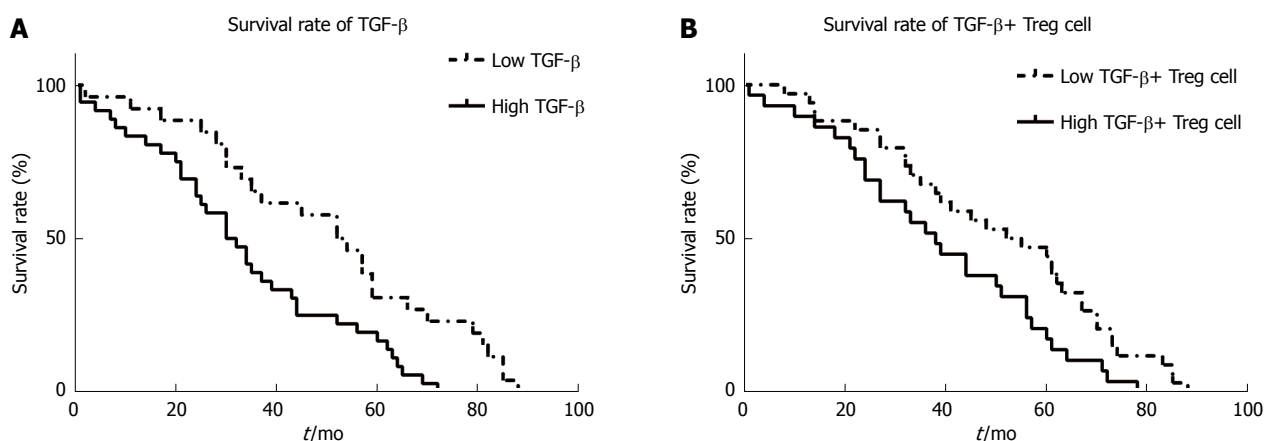

Figure 3 Level of transforming growth factor-β and transforming growth factor-β+ regulatory cells affect 5-year survival for hepatocellular carcinoma patients. The 5-year survival rates for HCC patients with a high and low level of TGF-β (A), and TGF-β+ Treg cells (B) in their PBMCs. The survival rates were determined using the Kaplan-Meier method (log-rank test). TGF-β: Transforming growth factor-β; Treg: Regulatory cells; HCC: Hepatocellular carcinoma.

in control serum samples. GP73 has high specificity and sensitivity for diagnosing HCC and is expected to be validated as another liver cancer marker[23]. The expression levels of des-gamma-carboxy prothrombin

(DCP) are affected by the pathological type and stage of tumors and the type of hepatic lesions in HCC. Its ability to diagnose HCC is better than that of alpha fetoprotein (AFP) and AFP-L3, which are first-line clinical tumor
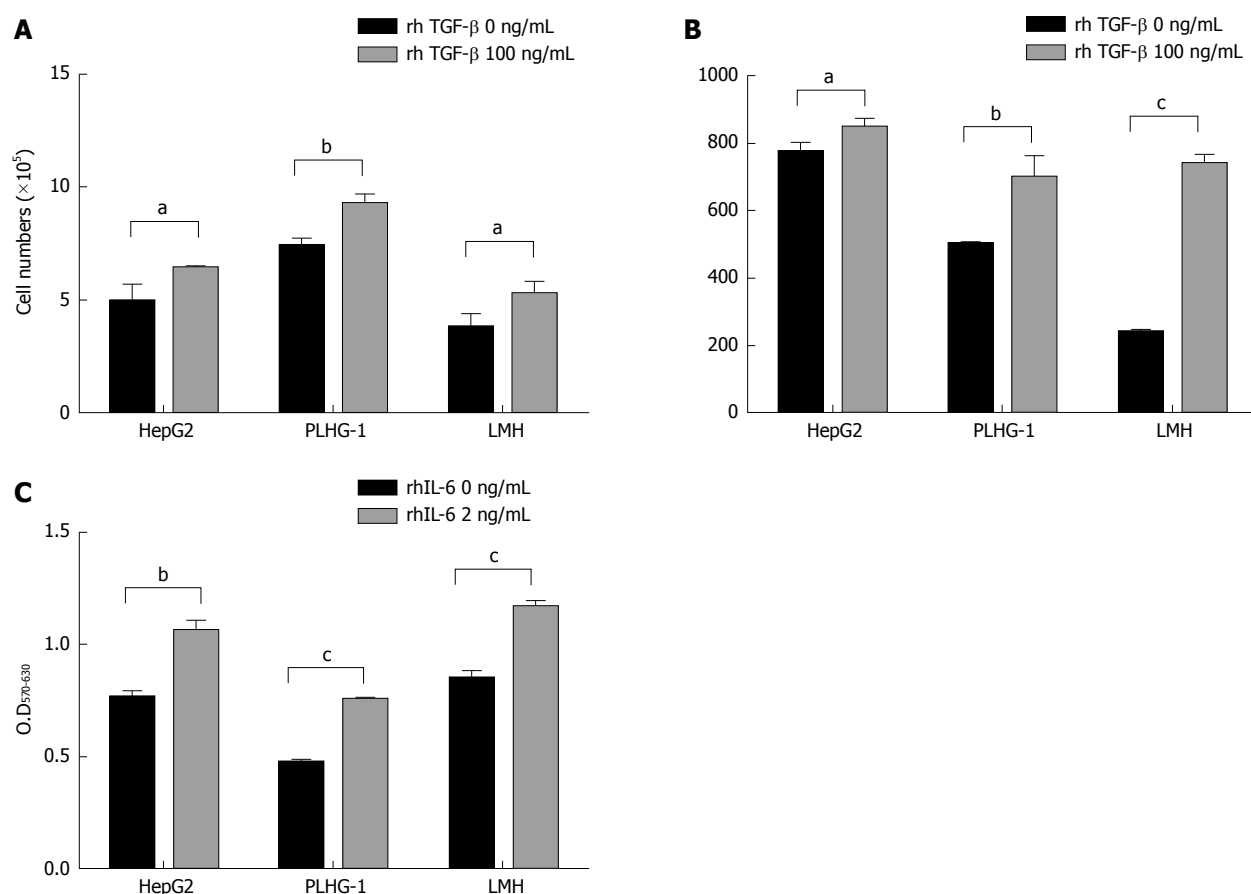
Figure 4 Transforming growth factor-β can promote the proliferation of tumor cells and increase the level of interleukin-6 in hepatocellular carcinoma cell lines production. A: The cell numbers of the three HCC cell lines after treatment with 100 ng/mL rhTGF-β for 48 h; B: The three HCC culture supernatants were analyzed for protein levels of IL-6 after treatment with rhTGF-β for 48 h using ELISA; C: The three HCC cell lines were treated with or without rhIL-6 for 48 h, and cell proliferation was determined by MTT assay. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$. TGF-β: Transforming growth factor-β; Treg: Regulatory cells; HCC: Hepatocellular carcinoma.

markers, and it can be used as an early indicator for diagnosing liver cancer[24,25].

Tregs are a subpopulation of T lymphocytes that have unique *in vivo* functions; Tregs can secrete IL-4, IL-10, and TGF-β and inhibit effector T cells, and they are involved in autoimmune diseases, transplantation immunity, and tumor immunity[26]. According to the biological function of Th cells and Tregs, there is a dynamic balance between the two types of immune cells. The disruption of the balance between proinflammatory Th17 cells and suppressor Tregs is a key factor in the development of inflammation, autoimmune diseases, and tumors, and their expression levels in different immune response types are not the same. Recent studies have shown that tumor cells secrete multiple factors that can induce the production of Tregs[27,28]. Th17 cells are negatively correlated with tumor progression because of the reduction in their number and proportion, whereas Tregs are positively correlated since their quantity and proportion are increased[29]; these data are consistent with the findings of our study. Tregs can suppress immune surveillance during tumor development in a mouse model of carcinogen-induced sarcoma[30]. Tregs can also be considered as the main component of tumor

evasion of the host immune system and, thus, can serve as an indicator of poor prognosis and even as a target for immunotherapy[31-33].

In summary, our study demonstrated that the levels of expression of TGF-β and TGF-β+ Tregs in the peripheral blood of HCC patients were significantly increased and were related to the progression of HCC and the 5-year survival of patients. This suggests that peripheral blood TGF-β levels can serve as a potential indicator of poor prognosis for HCC and provide a more effective method for the diagnosis and treatment of HCC.

ARTICLE HIGHLIGHTS

Research background
Although the treatment of hepatocellular carcinoma (HCC) has been improved, including surgery, chemotherapy, radiation therapy, and ablation, prognosis remains poor because of the recurrence and tumor metastasis.

Research motivation
Understanding the immune status, especially the inflammatory state, of cancer may provide insight into the development of new immunomodulation methods for the treatment of cancer and may potentially provide novel prognostic predictors of HCC.

Research objectives

To understand the cellular and molecular changes in peripheral blood that can lead to the development of HCC and provide new methods for its diagnosis and treatment.

Research methods

Peripheral blood mononuclear cells were isolated from peripheral blood of HCC patients and normal controls and then analyzed by flow cytometry. The percentage of transforming growth factor-β (TGF-β)+ Treg cells in peripheral blood was measured, and the expression of TGF-β was also detected. The relationship between the changes and the 5-year survival of patients was analyzed. In addition, recombinant human TGR-β and recombinant human IL-6 were added respectively to stimulate the cultured cells to evaluate its effect on HCC.

Research results

The expression of TGF-β and the percentage of TGF-β+ Treg cells in peripheral blood in HCC patients increased significantly compared with normal controls. Compared with the low TGF-β expression group, the 5-year survival rate was significantly lower in the high TGF-β expression group, and the same result was found in the two TGF-β+ Treg groups, suggesting that TGF-β and TGF-β+ Treg cells were negatively correlated with the overall survival of the patients. In addition, recombinant human TGF-β promoted the growth of tumor cells and induced high expression of IL-6 which can further promote tumor proliferation.

Research conclusions

The results showed that TGF-β may promote tumor growth and proliferation by inducing the production of IL-6, and TGF-β and TGF-β+ Treg cells may serve as new markers for predicting poor prognosis of HCC.

Research perspectives

Our study provided a novel insight to understand the cellular and molecular changes in peripheral blood of HCC and provide new methods for its diagnosis and treatment.

REFERENCES

1   **Llovet JM**, Villanueva A, Lachenmayer A, Finn RS. Advances in targeted therapies for hepatocellular carcinoma in the genomic era. *Nat Rev Clin Oncol* 2015; **12**: 436 [PMID: 26099984 DOI: 10.1038/nrclinonc.2015.121]

2   **Han KH**, Kudo M, Ye SL, Choi JY, Poon RT, Seong J, Park JW, Ichida T, Chung JW, Chow P, Cheng AL. Asian consensus workshop report: expert consensus guideline for the management of intermediate and advanced hepatocellular carcinoma in Asia. *Oncology* 2011; **81** Suppl 1: 158-164 [PMID: 22212951 DOI: 10.1159/000333280]

3   **Jemal A**, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90 [PMID: 21296855 DOI: 10.3322/caac.20107]

4   **Maluccio M**, Covey A. Recent progress in understanding, diagnosing, and treating hepatocellular carcinoma. *CA Cancer J Clin* 2012; **62**: 394-399 [PMID: 23070690 DOI: 10.3322/caac.21161]

5   **Ganss R**, Hanahan D. Tumor microenvironment can restrict the effectiveness of activated antitumor lymphocytes. *Cancer Res* 1998; **58**: 4673-4681 [PMID: 9788621]

6   **Coussens LM**, Werb Z. Inflammation and cancer. *Nature* 2002; **420**: 860-867 [PMID: 12490959 DOI: 10.1038/nature01322]

7   **Wherry EJ**. T cell exhaustion. *Nat Immunol* 2011; **12**: 492-499 [PMID: 21739672]

8   **Chen X**, Liang H, Guan D, Wang C, Hu X, Cui L, Chen S, Zhang C, Zhang J, Zen K, Zhang CY. A combination of Let-7d, Let-7g and Let-7i serves as a stable reference for normalization of serum microRNAs. *PLoS One* 2013; **8**: e79652 [PMID: 24223986 DOI: 10.1371/journal.pone.0079652]

9   **Beatty GL**, Gladney WL. Immune escape mechanisms as a guide for cancer immunotherapy. *Clin Cancer Res* 2015; **21**: 687-692

[PMID: 25501578 DOI: 10.1158/1078-0432.CCR-14-1860]

10   **Lin WW**, Karin M. A cytokine-mediated link between innate immunity, inflammation, and cancer. *J Clin Invest* 2007; **117**: 1175-1183 [PMID: 17476347 DOI: 10.1172/JCI31537]

11   **Douglas WG**, Tracy E, Tan D, Yu J, Hicks WL Jr, Rigual NR, Loree TR, Wang Y, Baumann H. Development of head and neck squamous cell carcinoma is associated with altered cytokine responsiveness. *Mol Cancer Res* 2004; **2**: 585-593 [PMID: 15498933]

12   **Linehan DC**, Goedegebuure PS. CD25+ CD4+ regulatory T-cells in cancer. *Immunol Res* 2005; **32**: 155-168 [PMID: 16106066 DOI: 10.1385/IR:32:1-3:155]

13   **Shi C**, Zhang Y, Yang H, Dong T, Chen Y, Xu Y, Yang X, Liu P. Ultrasound-targeted microbubble destruction-mediated Foxp3 knockdown may suppress the tumor growth of HCC mice by relieving immunosuppressive Tregs function. *Exp Ther Med* 2018; **15**: 31-38 [PMID: 29387180 DOI: 10.3892/etm.2017.5421]

14   **Glebezdina NS**, Olina AA, Nekrasova IV, Kuklina EM. Role of Endogenous Melatonin in the Regulation of Th17/Treg Balance during Pregnancy. *Bull Exp Biol Med* 2018; **164**: 462-465 [PMID: 29504099 DOI: 10.1007/s10517-018-4013-1]

15   **Patman G**. Liver cancer: TGF-β and cholangiocarcinoma. *Nat Rev Gastroenterol Hepatol* 2016; **13**: 2-3 [PMID: 26669764 DOI: 10.1038/nrgastro.2015.218]

16   **Achyut BR**, Yang L. Transforming growth factor-β in the gastrointestinal and hepatic tumor microenvironment. *Gastroenterology* 2011; **141**: 1167-1178 [PMID: 21839702 DOI: 10.1053/j.gastro.2011.07.048]

17   **Derynck R**, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003; **425**: 577-584 [PMID: 14534577 DOI: 10.1038/nature02006]

18   **Shen Y**, Wei Y, Wang Z, Jing Y, He H, Yuan J, Li R, Zhao Q, Wei L, Yang T, Lu J. TGF-β regulates hepatocellular carcinoma progression by inducing Treg cell polarization. *Cell Physiol Biochem* 2015; **35**: 1623-1632 [PMID: 25824460 DOI: 10.1159/000373976]

19   **Kryczek I**, Banerjee M, Cheng P, Vatan L, Szeliga W, Wei S, Huang E, Finlayson E, Simeone D, Welling TH, Chang A, Coukos G, Liu R, Zou W. Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. *Blood* 2009; **114**: 1141-1149 [PMID: 19470694 DOI: 10.1182/blood-2009-03-208249]

20   **Ikeda Y**, Kajiyama K, Yamashita Y, Ikegami T, Uchiyama H, Soejima Y, Kawanaka H, Ikeda T, Morita M, Oki E, Saeki H, Suehiro T, Mimori K, Sugimachi K, Shirabe K, Maehara Y. Differential expression of insulin-like growth factor 1 in human primary liver cancer. *Fukuoka Igaku Zasshi* 2013; **104**: 334-338 [PMID: 24511663]

21   **Wong VW**, Yu J, Cheng AS, Wong GL, Chan HY, Chu ES, Ng EK, Chan FK, Sung JJ, Chan HL. High serum interleukin-6 level predicts future hepatocellular carcinoma development in patients with chronic hepatitis B. *Int J Cancer* 2009; **124**: 2766-2770 [PMID: 19267406 DOI: 10.1002/ijc.24281]

22   **Mukozu T**, Nagai H, Matsui D, Kanekawa T, Sumino Y. Serum VEGF as a tumor marker in patients with HCV-related liver cirrhosis and hepatocellular carcinoma. *Anticancer Res* 2013; **33**: 1013-1021 [PMID: 23482775]

23   **Mao Y**, Yang H, Xu H, Lu X, Sang X, Du S, Zhao H, Chen W, Xu Y, Chi T, Yang Z, Cai J, Li H, Chen J, Zhong S, Mohanti SR, Lopez-Soler R, Millis JM, Huang J, Zhang H. Golgi protein 73 (GOLPH2) is a valuable serum marker for hepatocellular carcinoma. *Gut* 2010; **59**: 1687-1693 [PMID: 20876776 DOI: 10.1136/gut.2010.214916]

24   **Marrero JA**, Su GL, Wei W, Emick D, Conjeevaram HS, Fontana RJ, Lok AS. Des-gamma carboxyprothrombin can differentiate hepatocellular carcinoma from nonmalignant chronic liver disease in american patients. *Hepatology* 2003; **37**: 1114-1121 [PMID: 12717392 DOI: 10.1053/jhep.2003.50195]

25   **Volk ML**, Hernandez JC, Su GL, Lok AS, Marrero JA. Risk factors for hepatocellular carcinoma may impair the performance of biomarkers: a comparison of AFP, DCP, and AFP-L3. *Cancer Biomark* 2007; **3**: 79-87 [PMID: 17522429]

26   **Ernerudh J**, Berg G, Mjösberg J. Regulatory T helper cells in pregnancy and their roles in systemic versus local immune tolerance. *Am J Reprod Immunol* 2011; **66** Suppl 1: 31-43 [PMID: 21726336 DOI: 10.1111/j.1600-0897.2011.01049.x]

27   **Maturu P**, Jones D, Ruteshouser EC, Hu Q, Reynolds JM, Hicks J, Putluri N, Ekmekcioglu S, Grimm EA, Dong C, Overwijk WW. Role of Cyclooxygenase-2 Pathway in Creating an Immunosuppressive Microenvironment and in Initiation and Progression of Wilms' Tumor. *Neoplasia* 2017; **19**: 237-249 [PMID: 28254151 DOI: 10.1016/j.neo.2016.07.009]

28   **Holmgaard RB**, Zamarin D, Li Y, Gasmi B, Munn DH, Allison JP, Merghoub T, Wolchok JD. Tumor-Expressed IDO Recruits and Activates MDSCs in a Treg-Dependent Manner. *Cell Rep* 2015; **13**: 412-424 [PMID: 26411680 DOI: 10.1016/j.celrep.2015.08.077]

29   **Chen X**, Du Y, Lin X, Qian Y, Zhou T, Huang Z. CD4+CD25+ regulatory T cells in tumor immunity. *Int Immunopharmacol* 2016; **34**: 244-249 [PMID: 26994448 DOI: 10.1016/j.intimp.2016.03.009]

30   **Betts G**, Twohig J, Van den Broek M, Sierro S, Godkin A, Gallimore A. The impact of regulatory T cells on carcinogen-induced sarcogenesis. *Br J Cancer* 2007; **96**: 1849-1854 [PMID: 17565340 DOI: 10.1038/sj.bjc.6603824]

31   **Shou J**, Zhang Z, Lai Y, Chen Z, Huang J. Worse outcome in breast cancer with higher tumor-infiltrating FOXP3+ Tregs : a systematic review and meta-analysis. *BMC Cancer* 2016; **16**: 687 [PMID: 27566250 DOI: 10.1186/s12885-016-2732-0]

32   **Xu W**, Liu H, Song J, Fu HX, Qiu L, Zhang BF, Li HZ, Bai J, Zheng JN. The appearance of Tregs in cancer nest is a promising independent risk factor in colon cancer. *J Cancer Res Clin Oncol* 2013; **139**: 1845-1852 [PMID: 24005418 DOI: 10.1007/s00432-013-1500-7]

33   **Mizukami Y**, Kono K, Kawaguchi Y, Akaike H, Kamimura K, Sugai H, Fujii H. Localisation pattern of Foxp3+ regulatory T cells is associated with clinical behaviour in gastric cancer. *Br J Cancer* 2008; **98**: 148-153 [PMID: 18087278 DOI: 10.1038/sj.bjc.6604149]

SYSTEMATIC REVIEWS

Current global trends in the incidence of pediatric-onset inflammatory bowel disease

Josef Sýkora, Renáta Pomahačová, Marcela Kreslová, Dominika Cvalínová, Přemysl Štych, Jan Schwarz

Josef Sýkora, Renáta Pomahačová, Marcela Kreslová, Dominika Cvalínová, Jan Schwarz, Division of Gastroenterology, Hepatology and Nutrition, Department of Paediatrics, Charles University in Prague, Faculty of Medicine in Pilsen, Faculty Hospital, Pilsen 304 60, Czech Republic

Přemysl Štych, Department of Applied Geoinformatics and Cartography, Charles University in Prague, Faculty of Science, Prague 128 43, Czech Republic

ORCID number: Josef Sýkora (0000-0002-1256-4570); Renáta Pomahačová (0000-0002-7952-1364); Marcela Kreslová (0000-0001-7445-0942); Dominika Cvalínová (0000-0002-7246-8353); Přemysl Štych (0000-0002-9548-0777); Jan Schwarz (0000-0003-4214-8858).

Author contributions: Sýkora J and Schwarz J contributed equally to this work, and were responsible for the conception, the study design, the integrity of the data and the accuracy of the data analysis and performed the data acquisition; Pomahačová R, Kreslová M, Cvalínová D and Štych P analyzed the data, drafted the manuscript and revised it critically for important intelectuall content; Štych P performed and edited choropleth maps; Sýkora J and Schwarz J wrote the manuscript; all authors have seen and approved the version of manuscript to be submitted.

Conflict-of-interest statement: The authors have no conflicts of interest to disclose.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Abstract

AIM
To perform a comprehensive review and provide an up-to-date synopsis of the incidence and trends of inflammatory bowel disease (IBD).

METHODS
We systematically searched the MEDLINE (source PubMed), EMBASE and Cochrane Library databases in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (period: 1985-2018) to identify studies reporting population-based data on the incidence of pediatric-onset (< 19 years at diagnosis) IBD in full manuscripts. Two authors carried out screening and data extraction. Choropleth interactive maps and temporal trends were used to illustrate the international differences and incidences of and changes in IBD and subtypes.

RESULTS

In total, one hundred forty studies reporting data from 38 countries were considered in this review. The highest annual pediatric incidences of IBD were 23/100000 person-years in Europe, 15.2/100000 in North America, and 11.4/100000 in Asia/the Middle East and Oceania. The highest annual incidences of Crohn's disease (CD) were 13.9/100000 in North America and 12.3/100000 in Europe. The highest annual incidences of ulcerative colitis (UC) were 15.0/100000 in Europe and 10.6/100000 in North America. The highest annual incidences of IBD-unclassified (IBD-U) were 3.6/100000 in Europe and 2.1/100000 in North America. In the time-trend analyses, 67% of CD, 46% of UC and 11% of IBD-U studies reported an increasing incidence ($P < 0.05$). The risk of IBD is increasing among first-generation of migrant populations.

CONCLUSION

Globally, the incidence of IBD varies greatly by geographical areas. The steadily increasing incidence of pediatric IBD over time indicates its emergence as a global disease, suggesting that studies should investigate the environmental risk factors among pediatric cohorts.

Key words: Children; Incidence; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Inflammatory bowel disease-unclassified

Core tip: The incidence of inflammatory bowel disease (IBD) is unclear in the pediatric literature. We comprehensively reviewed and critically evaluated population-based and national cohort studies investigating the incidence of IBD and its global trends. One hundred forty studies met the inclusion criteria. The incidence of pediatric-onset IBD has been steadily increasing over time in different geographical areas in both developed and developing regions worldwide, whereas those in the West may have reached a plateau. This indicates the emergence of an IBD epidemic; however, incidence data from developing regions are limited. Exploring the changes and increasing incidence of pediatric IBD may provide new insights into the potential etiology of IBD.

INTRODUCTION

Inflammatory bowel disease (IBD) encompasses the following three forms of idiopathic inflammation of the gut: ulcerative colitis (UC), Crohn's disease (CD) and IBD-unclassified (IBD-U). The differentiation of IBD-U from CD and UC remains difficult; thus, the incidence of IBD-U must be explored. Approximately 25% of patients first present symptoms before the age of 18 years[1], and the incidence in children over 10 years of age is clearly increasing[2]. The incidence of IBD in industrialized countries is higher than that in developing countries; however, overwhelming data suggest that its prevalence is increasing worldwide in both adult[3] and pediatric populations[4] and that its distribution is uneven among regions[5]. Many explanations have been proposed, but the hypothesis that exposure to environmental and genetic factors is a fundamental contributor to the development of IBD has been challenged by several new observations[6-10]. Whether the etiology of pediatric-onset IBD differs from that of adult-onset IBD remains unknown[11]. Thus, there is a great need to summarize global information regarding the pediatric IBD incidence and disease burden in different settings and perform subsequent analyses of the underlying factors.

The aim of this review is to delineate the incidence of pediatric IBD (defined as onset at an age < 19 years) and summarize the latest incidence trends based on a comprehensive review of credible studies.

MATERIALS AND METHODS

Search and study selection

We conducted a systematic literature search according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)[12]. Articles were identified using computer-stored databases and manual search. A search of English and non-English language journals in the MEDLINE (source PubMed), EMBASE *via* OvidSP, and Cochrane Library databases was conducted to identify original studies investigating IBD incidence published between January 1, 1985 and March 2018. Suitable papers were subsequently catalogued. The Cochrane library was reviewed. We searched for the following strings according to the MeSH headings: "pediatric"[MeSH Terms] and "inflammatory bowel disease"[All Fields] or "Crohn´s disease"[All Fields] or "ulcerative colitis"[All Fields] or "inflammatory bowel disease unclassified"[All Fields] or "indeterminate colitis" [All Fields] and "incidence"[All Fields] or "children"[MeSH terms] or "adolescents"[MeSH terms] and "population-based studies"[All Fields] or "national registries"[All Fields] or "health administrative database"[All Fields] and "individual country names"[MeSH terms]. The detailed search strategy is shown in Figure 1.

Data extraction

The data extraction was performed independently by two researchers (Schwarz J and Sýkora J) using set criteria to analyze the title and abstract and extract all relevant study-specific data required for the analysis.
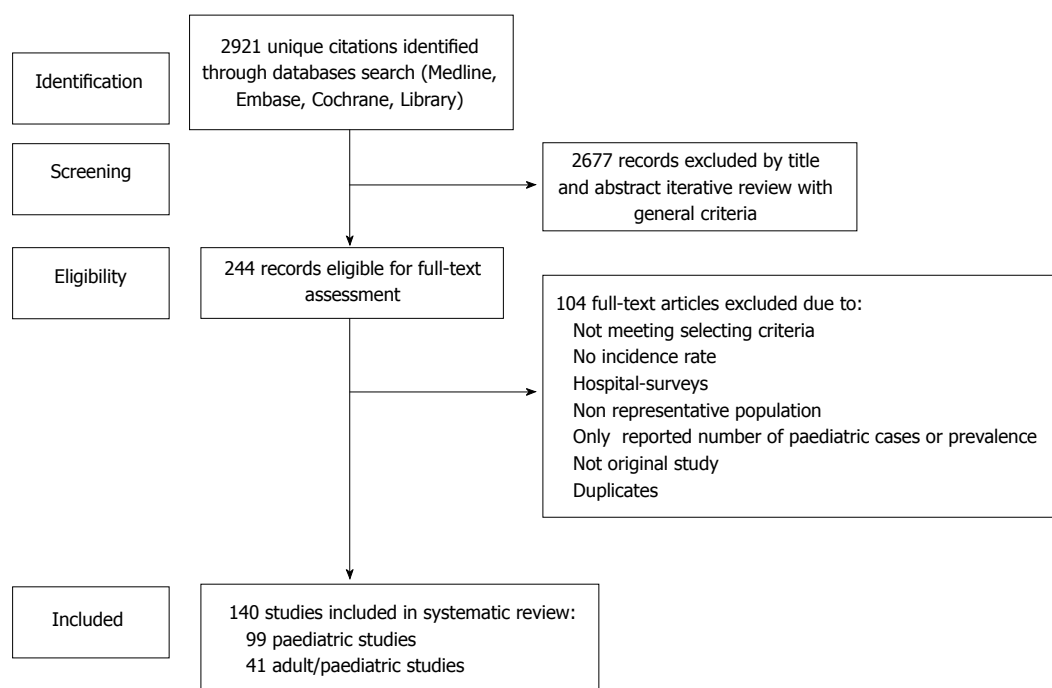
```
Identification        2921 unique citations identified
                      through databases search (Medline,
                      Embase, Cochrane, Library)

Screening                                               →  2677 records excluded by title
                                                           and abstract iterative review with
                                                           general criteria

Eligibility           244 records eligible for full-text
                      assessment
                                                           104 full-text articles excluded due to:
                                                              Not meeting selecting criteria
                                                              No incidence rate
                                                              Hospital-surveys
                                                              Non representative population
                                                              Only  reported number of paediatric cases or prevalence
                                                              Not original study
                                                              Duplicates

Included               140 studies included in systematic review:
                          99 paediatric studies
                          41 adult/paediatric studies
```

Figure 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses flowchart diagram for study selection.

This strategy was performed iteratively until no more relevant publications were found. During the full-text screening stage, we analyzed papers for inclusion suitability and adequate information regarding the incidence of IBD or calculation of incidence, and a final decision was made about inclusion/exclusion. In addition, the references in the relevant articles were checked by the first author. From each study, the data were extracted and sorted by the following variables: name of the study, leading author, journal, publication year, study period, type of study, age, study location, crude incidence rate and time trends. Double-extracted data were verified, when a disagreement occurred related to data extraction, this was resolved by consensus. Attempts were made to clarify missing data and any uncertainties or errors found in the studies by consulting with the corresponding authors. The rates were extrapolated from the figures if the articles reported data without specifying the numeric rates. The average incidence was calculated if the incidence rates were reported separately for females and males or by race/ethnicity. Reports focusing on immigrant/indigenous populations were analyzed separately based on the respective general population of the area. After the selection of appropriate articles extrapolating the incidence of IBD, a comprehensive review was performed. We only included original studies with a clear case ascertainment. A meta-analysis was not undertaken due to the variability in the included records; thus, a narrative review was conducted. The incidence was measured as the number of cases/100000 person-years, and the temporal evolution was examined to measure the effect of the calendar year on the incidence. All studies were organized by world and regional distribution and subsequently analyzed based on the United Nations (UN) geoscheme devised by the UN Statistics Division[13] regarding the assignment of countries or areas to specific groupings as follows: North America, Europe, Oceania, Asia, South America and Africa. We created interactive (choropleth) maps to visualize the resulting data worldwide and extrapolate the incidence at the country level. Choropleth maps depicting differences in the incidence rates by changes in color were employed. If data were reported for only a region within a country, the entire country was shown on the map.

Quality assessment of selected papers
The quality of the incidence studies was assessed by whether the diagnostic criteria were clearly defined or recognized criteria (Lennard-Jones, Copenhagen criteria) were implemented[14]. The Critical Appraisal Skills Programme was used to appraise the principles of the studies based on the specific methodological designs of the included studies[15]. We extrapolated the incidence at the country level and summarized the incidence rates by jurisdiction in each study. The quality characteristics of each paper are summarized in Table 1. The assessment of the studies yielded either an A or B quality ranking based on the representativeness of the cases. A representative study population was a prerequisite for a study to be included in this systematic review (A). Studies that included populations that were not sampled across the entire country or had a smaller population size were given a B quality ranking. Because all papers were considered to contribute to the determination of the incidence and differences in incidence estimates, we included all studies without a

Table 1 Summary of included pediatric-onset inflammatory bowel disease incidence studies stratified by continent and geographical areas

Country/Region/Province	Age (yr)	Study period	IBD incidence	CD incidence	UC incidence	IBDU incidence	Setting	Year of publication	First author /reference	Representativeness of the case
Western and North Europe										
Austria/Styria	< 18	1997-2007		6.7 a	4.8a		R	2013	Petritsch W *et al*[122]	B
Denmark/Eastern	< 15	1998-2000	4.3.	2.3.	1.8.	0.2	R	2002	Urne *et al*[108]	B
Denmark	< 17	1995-2013					P	2016	Larsen *et al*[109]	A
		2013		9.7a	6.7a					
Denmark/Eastern	< 15	1998-2009	6.4a	3.2a,b	3.1a,b	0.2a	R	2011	Jakobsen *et al*[110]	B
Denmark	< 15	1995-2012		3.1a	2.7a		P	2014	Norgard *et al*[111]	A
Denmark/Eastern	< 15	1998-2000	4.3a	2.3a	1.8a	0.2a	P	2008	Jakobsen *et al*[33]	B
		2002-2004	6.1	3.1	2.7	0.3				
Denmark	< 15	1980-2013		2.4a	3.3a		P	2017	Lophaven *et al*[103]	A
Denmark/Copenhagen County	< 19	1962-1987			8.6-13.3a		P	1991	Langholz *et al*[106]	B
Denmark/Copenhagen County	< 14	1962-1987	2.2a	0.2a	2.0a		P	1997	Langholz *et al*[105]	B
Denmark/Copenhagen County	< 14	1962-1987			0.2		R	1992	Munkholm *et al*[62]	B
Denmark/Copenhagen County	< 15	1962-1987		0.2-3.1a	2 - 1.6a		P	2009	Jakobsen *et al*[34]	B
Denmark	< 15	1981-1992		0.8a	2.5a		P	1997	Fonager *et al*[107]	A
Denmark/Northern	< 14	1978-2002		1.5	2.7		P	2006	Jacobsen *et al*[104]	B
Denmark/Copenhagen County	< 17	2003-2005		4.4	5.5		P	2006	Vind *et al*[102]	B
Finland	< 18	1987-2003	5a	2a	4a		P	2011	Lehtinen *et al*[65]	A
		2003	15	5	9.1					
Finland	< 17	1987-2003					P	2006	Turunen *et al*[64]	A
		1987	3.9a	1.7a	2.2a					
		2003	7	3.6	4.8	1				
Finland	< 17	1986-1992		1.0a			R	1998	Pebody *et al*[88]	A
Finland	< 18	1987-2003	6.5	2.1	4.1		P	2016	Lehtinen *et al*[89]	A
Finland	< 19	1987-2014	7-23a	6-8a	10-15a		R	2017	Virta *et al*[66]	A
	< 16	2011-2014	13		8					
France	< 17	1988-2011	3.1-6.3a				P	2016	Bequet *et al*[126]	A
France/Northern	< 17	1998-1999	3.1a	2.3a	0.8a	0.12a	P	2005	Auvin *et al*[43]	B
France/Northern	< 17	1988-2011	4.4a	3.2a	1.1a	0.1a	P	2017	Ghione *et al*[131]	B
France/Brittany	< 17	1994-1997	2.5	1.6	0.57		P	2000	Tourtelier *et al*[131]	B
France/Northern	< 19	1988-2007		3.4-5.9a			P	2011	Chouraki *et al*[32]	B
France/Northern	< 17	1988-2002		2.6			P	2008	Vernier-Massouille *et al*[127]	B
France/Northern	< 19	1988-2008		6.5-12.9a			P	2013	Gower-Rousseau *et al*[125]	B
France/Corsica	< 19	2002-2003		12.3	6.2		P	2007	Abakar-Mahamat *et al*[128]	B
France/Nord-Pas-de-Calais	< 17	1988-1989	3.1	2.1	0.5		P	1991	Gottrand *et al*[130]	B
The Farose Islands	< 19	1960-2014	9.0²a	2.5²a	5²a	1.5²a	P,R	2016	Hammer *et al*[19]	A
Iceland	< 16	1950-2010					P,R	2013	Agnarsson *et al*[29]	A
		1951-1960	1.2	0.2	1.1					
		1961-1970	0.9	0.1	0.7					
		1971-1980	0.9	0.1	0.7					
		1981-1990	2.5a	1.2a	1.2a	0.1a				
		1991-2000/2001-2010	5.6/5.0	2.5/2.3	2.5/2.3	0.3/0.3				
Iceland	< 19	1990-1994		4.5	5		P	2000	Bjornsson *et al*[112]	A

Country/Region	Age	Period	IBD	CD	UC		P/R	Year	Author	Class
Ireland	< 16	2000-2010	2.5-5.6a	2.3a	1.1a		R	2012	Hope et al[120]	A
Ireland	< 10	2000-2014	0.8-3.3a				P,R	2017	Coughlan et al[121]	A
The Netherlands	< 18	1999-2001	5.2	2.1	1.6	3.6	P	2004	van der Zaag-Loonen et al[124]	A
Germany/Southern	< 15	2004-2006	4.0a	2.4a	1.1a		P	2008	Ott et al[123]	B
Norway/Southeastern	< 16	1990-1994	4.2	2	2.1		P	2002	Bentsen et al[85]	B
Norway/Southeastern	< 14	1990-1993			1.3		P	1996	Moum et al[95]	B
Norway/Southeastern	< 16	1990-1993	4.7	2.7	2		P	2004	Stordal et al[93]	B
Norway/Southeastern	< 18	2005-2007	10.9b	6.8b	3.6b	0.6	P	2009	Perminow et al[92]	B
Norway/Southeastern	< 16	1993-2004	5.6-5.7a	2.0-3.6a	3.7-2.1a		P,R	2006	Perminow et al[60]	B
Norway/Western	< 16	1984-1985	6.8	2.5	4.3	0	P	1989	Olafsdottir et al[97]	B
Norway/Western	< 14	1984-1985		2.5			P	1988	Haug et al[163]	B
Norway/Souteastern	< 14	1990	3	1.2	0.6		P	1995	Moum et al[94]	B
Norway/Southeastern	< 14	1990-1993		0.94			P	1996	Moum et al[96]	B
Sweden/Northern Stockholm	< 17	1990-1998	6.9a	3.8a	2.1a	1.1a	R	1999	Askling et al[99]	B
Sweden/Northern Stockholm	< 15	1990-2001	7.4a	4.9a	2.2a	0.2a	P	2003	Hildebrand et al[59]	B
Sweden/Soutwestern	< 16	1983-1987	5.3	2.7	2.6	0.7	P	1994	Hildebrand et al[164]	B
Sweden/Stockholm	< 15	2002-2007	12.8a,b	9.2a,b	2.8a,b		P	2013	Malmborg et al[38]	B
Sweden	< 15	1984-1995	4.6-7.0a	1.2-1.3a	1.4-3.2a	1.1a	P	2000	Lindberg et al[68]	A
Sweden	< 14	1963-1967		1.4 - 0.7a			R	1991	Lindberg et al[98]	A
		1983-1987		0.7						
Sweden/the Uppsala region	< 17	2005-2009	18.9		8.9		P	2013	Sjoberg et al[100]	B
Sweden/the Uppsala region	< 17	2005-2009		10			P	2014	Sjoberg et al[101]	B
Sweden/the Uppsala region	< 19	1965-1983					R	1991	Ekbom et al[56]	B
		1965-1970		5.9-5.0a	5.2-4.8a					
		1975-1983		4.8-3.2	3.9-2.7					
Sweden/Orebro	< 19	1963-1987			6		R	1992	Tysk et al[165]	B
Sweden/Stockholm County	< 14	1955-1983		4.1			R	1997	Lapidus et al[35]	B
Sweden	< 15	1984-1985	4.8	1.7	1.7		P	1991	Hildebrand et al[166]	A
UK/ Scotland	< 16	2003-2008	7.8a	4.8a	2.1a	1.0a	P,R	2012	Henderson et al[2]	A
		1990-1995	4.5	2.9	1.6					
UK/Wesex Southern England	< 16	2002-2012/2013-2017						2014, 2018	Ashton et al Ashton et al[116,117]	B
		2002-2006	6.4a,b	3.8a,b	2.0a,b		P			
		2008-2012	9.4	5.9	2.6					
UK/Scotland	< 16	1981-1995	3.4	2.3a	1.2a		R	2004	Armitage et al[114]	A
UK/Wales	< 16	1996-2003	5.4	3.6	1.5		P	2006	Ahmed et al[30]	B
UK/Wales	< 16	1995-1997	2.6	1.4	0.8	0.5	P	2000	Hassan et al[118]	B
UK/Wales	< 16	1981-1995		2.5a	1.3a		R	2001	Armitage et al[53]	B
UK/ Northeastern Scotland	< 16	1980-1999								
		1980-1989		2.2	0.7		R	2002	Watson et al[115]	B
		1990-1999		4.4	1.5					
UK/Scotland	< 16	1968-1983		0.7-2.3a	1.9-1.6a		R	1989	Barton et al[61]	A
UK/Cardiff	< 16	1996-2005		2.7			R	2007	Gunesh et al[119]	B

UK/Leicestershire	< 10	1972-1989			0.3a,d		R	1992	Probert et al[27]	B
UK/Wales South Glamorgan	< 16	1983-1993		2.2a	0.7a		R	1996	Cosgrove et al[57]	B
		1983-1988		1.3	0.7					
		1989-1993		3.1	0.7					
UK/British Isles and Ireland	< 16	1998-1999	5.3a,d	3.1a,d	1.4a,d	0.6d	P	2001	Sawczenko et al[16]	A
UK/Scotland	< 19	1990-1992		2.9a			R	1999	Armitage et al[113]	A
UK/East London	< 20	1997-2001		2.3-7.3a,d	2.4-8.1a,d		R	2004	Tsironi et al[17]	
Northern America										
Canada/Manitoba	< 17	1978-2007	6.9a	1.2-4.7a	0.5-1.6a		P	2014	El-Matary et[79]	B
Canada/Metropolitan Toronto	< 17	1991-1996		3.7	2.7		R	2004	Griffiths[82]	B
Canada/ 5 Provinces	< 16	1999-2010	9.7a	6.5a	2.4a		R	2017	Benchimol et al[31]	A
Alberta			9.7	5.9	2.7					
Manitoba			7.2	4.2	2.8					
Nova Scotia			15.2	9.3	4.2					
Ontario			9.3	5.5	3.2					
Quebec			10.3	8.8	1					
Canada/Suothwestern Ontario	< 17	1997-2006	13.3a	4.9a	8.1a	0.3a	R	2009	Grieci et al[81]	B
		1997-2001	14.3	3.5	10.6	0.2				
		2002-2006	12.4	6	6	0.4				
Canada/Ontario	< 18	1994-2005					P	2009	Benchimol et al[54]	B
		1994	9.5a	6.2a	4.4a					
		2005	11.4	7	4.8					
Canada/Ontario	< 19	1999-2008					P	2014	Benchimol et al[84]	B
	0-9		2.9a	1.3a	1.3a	0.3				
	10-19		21.5	12.8	7.6	1.1				
Canada/British Columbia	< 16	1985-2005	5.2d	3.7d	1.0d	0.5d	R	2007	Pinsk et al[18]	B
			15.2	6.4	6.7	2.1				
Canada/Ontario	< 18	1994-2009	9.4-13.2a	5.2-7.9a	3.9-4.1a		R	2014	Benchimol et al[84]	B
Canada/Eastern	< 20	1996-2009		14-11a²c	4-6a²c	0-1.5a²c	P	2014	Leddin et al[83]	B
Canada/Quebec	< 19	2001-2008					R	2014	Bitton et al[78]	B
	0-9			2.0a	1.0a					
	10-19			20	4					
Canada/ 5 Provinces	< 19	1998-2000					P	2006	Bernstein et al[39]	A
Alberta				9.4a	4.1a					
British Columbia				5.4	3.2					
Manitoba				6.9	4.5					
Nova Scotia				12	5.7					
Saskatchewan				7.9	4.2					
Canada/Qubec	< 19	1993-2002		13.9			P	2009	Lowe et al[80]	B
U.S.A./Northern Carolina	< 17	1996-2006		2.2-4.3a,d	1.8-4.9a,d	0.5d	R	2010	Abramson et al[20]	B
U.S.A/Wisconsin	< 18	2000-2001	7.05d	4.6d	2.1d		P	2003	Kugathasan et al[21]	B
U.S.A/Wisconsin	< 18	2000-2007	9.5a,d	6.6a,d	2.4a,d	0.5a,d	P,R	2013	Adamiak et al[22]	B
U.S.A/Georgia	< 19	1986-1995		8.8a,d	5.3a,d		R	1998	Ogunbi et al[23]	B
U.S.A/Texas	< 17	1991-2002					R	2010	Malaty et al[52]	B
		1991-1996	1.1a	0.7a	0.3a	0.1a				
		1997-2002	2.4	1.3	0.5	0.7				
U.S.A/Olmstedt Minesota	< 19	1940-2000		3.4a	2.4a		R	2007	Loftus et al[58]	B
		1990-2000		4.8	3.2					
U.S.A/Olmstedt Minesota	< 14	1943-1982		0.75a			R	1988	Gollop et al[167]	B
U.S.A/Olmstedt Minesota	< 19	1940-1993		2.5a			R	1998	Loftus et al[36]	B

Location	Age	Period					Type	Year	Author	
U.S.A/Northern California	< 18	1996-2002		3	2.9		R	2008	Herrinton et al[76]	B
U.S.A/Olmstedt Minesota	< 19	1940-1993			1.8		R	2000	Loftus et al[168]	B
U.S.A/Rhode Island	< 19	2008-2010					P	2016	Shapiro et al[77]	B
	0-9			4.5a	1.1a	0.0a				
	10-19			20.6	8.6	0.7				
Latin America										
Argentina/Provinces	< 18	2012-2013								
Argentina			0.4				P	2017	Vincentin et al[87]	A
Buenos Aires			0.3							
CABA			2.4							
Chaco			0.3							
Cordoba			0.3							
Corrientes			0.6							
Entre Rios			0.8							
Mendoza			0.2							
Misiones			0.9							
San Juan			1							
Tucana			0.2							
Africa										
Libya/Eastern/Benghazi	< 15	1997-2006					R	2009	Ahmaida et al[146]	B
		1997	0.0a							
		2000	0.2							
		2006	0.9							
Asia /Middle East										
Saudi Arabia	< 14	2003-2012	0.5	0.3	0.2		R	2014	El Mouzan et al[149]	A
Saudi Arabia/Riyadh	< 18	1993-2002	0.5				R	2006	El Mouzan et al[150]	B
Kuwait	< 15	1998-2008	2.2	1.5	0.6	0.03	R	2011	Al-Quabandi[151]	A
Israel/Tel Aviv	< 19	1970-1980			1		P	1989	Grossman et al[148]	B
Israel/Southeastern	< 19	1968-1992		3.6			R	1994	Odes et al[147]	B
the Kingdom of Bahrain	< 19	1990-2015		3.7a			P,R	2017	Zayyani et al[152]	A
		1990-1995		1.8						
		2010-2015		7.6						
Taiwan	< 18	1979-2000					R	2004	Tsai et al[154]	A
		1979-1995		0.9a	0.9a					
		1996-2000		2.6	1					
Singapore	< 18	1996-2009					R	2013	Chu et al[155]	A
		2000	2.2a							
		2008	11.4							
Taiwan	< 19	2000-2010					R	2015	Chia Jung Kuo et al[169]	A
	0-9			0.1a	0.1a					
	10-19			0.2	0.2					
Korea/South	< 19	2011-2014						2017	Jung et al[157]	B
	10-14			1.6[2]	2.0[2]		P			
	15-19			8.2[2]	4.8[2]					
China/Shangahai	< 18	2000-2010	5.5a	2.9a	2.5a		R	2013	Wang et al[153]	B
Korea/Seoul	< 19	1986-1997					R	2000	Yang et al[156]	B
	0-9	1986-1995/1995-1997			0-0.2					
Australasia	10-19	1986 -1995/1995-1997			0.2-0.9					
Australia/Randwick	< 16	1987-2011	33.1-4.3d				R	2014	Naidoo et al[24]	B
Australia/Victoria	< 16	1971-2001		0.1-2.0a			R	2003	Phavichitr et al[158]	B
Australia/Victoria	< 16	1983-1998		2.0a			R	2009	Ponsonby et al[37]	B
Australia/Victoria	< 16	1950-2009			0.4-1.6a		R	2013	Schildkraut et al[159]	B

Location	Age	Period	IBD	CD	UC	IBD-U	Type	Year	Ref	Group
New Zealand	< 16	2015	5.2	3.5	1	0.7	P	2017	Lopez et al[160]	A
New Zealand/Canterburry	< 16	1996-2015	7.2a				P	2018	Lopez et al[161]	B
New Zealand Sousthern	< 15	2002-2003	2.9d	1.9d	0.5d		P	2008	Yap et al[26]	A
Europe										
Sousthern Europe										
Italy/Florence	< 15	1978-1992		1.9-3.4a	3.8-9.6a		R	1996	Tralori et al[136]	B
Italy/Lazio	< 19	2008-2009					R	2014	Domenicantonio et al[135]	B
	0-9			2.5	2					
	10-19			8.72	7.52					
Italy	< 18	1996-2003	0.9-1.4a				P	2008	Castro et al[63]	A
Italy/Lombardia	< 14	1990-1993		1.2	1.2			1996	Ranzi et al[134]	B
Spain	< 18	1996-2009	1.0-2.8a,b	0.5-1.7a,b	0.4-0.9a,b		R	2013	Martin de Carpi et al[42]	A
Spain/the Vigo area	< 15	2010	18.3	10.3	8.7	1.2	P	2015	Fernandéz et al[50]	B
Spain/Navarra	< 14	2001-2003	2.6	1.7	0.9	0.6	P	2008	Letamendia et al[137]	B
Spain	< 18	1985-1995	0.2a[1]				R	2014	Martin de Carpi et al[51]	A
Spain/Asturias	< 14	1993-2000	0.3	0.2	0.1		P,R	2004	Fernandez Gonzalez[170]	B
Spain/Northern	< 14	2000-2002		5.8	1.6		P	2004	Rodrigo et al[44]	B
Spain/Aragon	< 14	1992-1995		0.4a	0.3a		P	1999	Lopez Miguel et al[171]	B
Malta	< 15	1993-2005		1.3a	7.9a		R	2008	Cachia et al[138]	A
Central and Eastern Europe										
Croatia/Primorsko-Goranska	< 18	2000-2004		8.7	0.9		P	2006	Sincic et al[47]	B
Czech Republic/Moravia	< 16	1998-2001	2.2a	2.7a	1.8a	0.3a	R	2004	Kolek et al[140]	B
Czech Republic	< 15	1990-2001		0.3-.1.3a			P,R	2006	Pozler et al[139]	A
Czech Republic/West Bohemia	< 19	2000-2015	10.0a	6.2a	2.8a	1.0a	P	2017	Schwarz et al[45]	B
	< 15		7.3	4.6	2	0.7	P			
Hungary	< 16	2007-2009	7.5	4.7	2.3	0.5	P	2013	Muller et al[141]	A
Hungary/Veszprem	< 19	1977-2011		0.0-7.2a	0.7-5.2a		P,R	2014	Lovasz et al[46]	B
Slovenia/Northestern	< 18	2002-2010	7.6	4.6	2.8		R	2014	Urlep et al[49]	B
		2002-2004	5.7	3.9	1.8					
		2008-2010	8.9	5	3.4					
Slovenia	< 18	2002-2010	7.6a,b	4.5a,b	2.9a,b	0.2a,b	R	2015	Urlep et al[48]	A
		2002-2004	5.8	3.6	2.2					
		2005-2007	8.6	5.1	3.2					
		2008-2010	8.4	4.9	3.2					
Slovenia/Western	< 17	1994-2005	4.0a	2.4a	1.1a	0.5a	R	2009	Orel et al[143]	B
		1994-1999	3	2	0.8	0.3				
		2000-2005	5.1	2.9	1.6	0.69				
Yugoslavia/Zagreb Croatia	< 14	1980-1989		1.76			P,R	1991	Vucelic et al[145]	B
Yugoslavia/Zagreb Croatia	< 14	1980-1989			3.1		P,R	1991	Vucelic et al[144]	B
Poland	< 18	2002-2004	2.7	0.6	1.3	0.8	P	2009	Karolewska-Bochenek et al[67]	A
Hungary/Western	< 18	1977-2008		7.5a	5.5a		P	2011	Lakatos et al[142]	B

[1]An approximate incidence rate at the beginning of the 25-year period based on the estimative population between the demographic census of 1981 and 1991; [2]The incidence rates were extrapolated from Figures. Incidence: Incidence rates per 100000 person-years; P: Prospective; R: Retrospective; P,R: Prospective/retrospective; a: Statistical significant for time trend analysis ($P < 0.05$); b: Study incorporated previously published cohort for comparison from the same geographical area the rest of pediatric population; c: The only one study under the age of 20 years d: Studies on race, ethnicity and migration compared to the rest of pediatric population. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; IBD-U: Inflammatory bowel disease-unclassified.

Table 2 Summary of range in pediatric-onset inflammatory bowel disease incidence stratified by continent and geographical regions

Regions	IBD incidence	UC incidence	CD incidence	IBD-U incidence	Time period
Europe					
North/West	0.5-23	0.3-15	0.2-12.3	0.2-3.6	1951-2017
East	2.7-10.0	0.9-5.2	0.25-8.6	0.3-1	1997-2015
South	0.1-18.3	0.1-9.6	0.5-10.3	0.6-1.2	1978-2005
North America	1.1-15.2	0.5-10.6	0.7-13.9	0.2-2.1	1940-2010
Latin America	0.2-2.4	NR	NR	NR	2012-2013
Africa	0.0-0.9	NR	NR	NR	1997-2006
Asia/Middle East	0.5-11.4	0.2-3.9	0.3-3.7	0.1	1968-2012
Australasia	2.9-7.3	0.4-1	0.1-3.5	0.7	1971-2015

NR: Not reported; Incidence: Incidence rates per 100000 person-years; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; IBD-U: Inflammatory bowel disease-unclassified.

quality assessment of each manuscript.

Summarization of data

Population-based or national/subnational cohort studies reporting incidence were included in the analysis of the temporal evolution of IBD. The inclusion criteria were as follows: (1) Population-based or national cohort/health care administrative database studies (a study was considered population-based if all residents within specific areas were included, and the study population was representative of the catchment area; studies were considered at the national level as stated in the report or if the study was multicenter involving multiple regions in a country, sub-national level if only a particular region was evaluated, or city level); (2) published full-text manuscripts; (3) studies defining pediatric patients as patients younger than 19 years of age; (4) studies describing patients in the entire age range; and (5) studies performed within the geographical regions outlined by the UN. The exclusion criteria were as follows: (1) Studies with a sample size < 5 patients; (2) studies that did not report original data (*e.g.*, review articles, meta-analyses, conference presentations, and guidelines); (3) studies that only demonstrated the incidence of adult-onset IBD (IBD onset after the age of 19 years); (4) studies reporting hospital surveys; (5) studies reporting only the number of pediatric cases (no incidence per population) and prevalence studies because our interest was disease incidence; (6) studies without defined study periods; and (7) duplicate studies reporting the same outcome in an identical cohort. To avoid selection bias due to cohort overlap, only the most recent study was included.

RESULTS

Study characteristics

In total, 140 papers were retrieved, and a significant proportion of the studies was conducted in European countries (96 in Europe, 23 in North America, and the remaining 21 in Latin America, Africa, Asia/Middle East and Oceania); overall, the studies reported the IBD incidence in 38 countries. Moreover, wide variability

in study design was observed. The upper age limit defining the pediatric population differed across the studies and ranged mostly between 15 and 19 years. The characteristics, distribution and detailed results of the 140 incidence studies, including references, are summarized in Table 1. As shown in Table 1, the ratio of CD to UC and IBD-U varied geographically. Of the included papers, 99 (71%) reported the IBD incidence in children, while the remaining 41 (29%) reported the rates of IBD in non-pediatric studies, but distinctions were made between adults and children in reporting the data. Of the 140 studies, the sample frame was prospective in 72 (51%) studies and retrospective in 57 (41%) studies. The data of the 11 (8%) studies were combined for this analysis.

Incidence of IBD

Broad variation was observed in the incidence rates, which ranged from 0.5 to 23/100000 for IBD, 0.1 to 13.9/100000 for CD, 0.3 to 15.0/100000 for UC, and 0.0 to 3.6/100000 for IBD-U. As shown in Table 2, the incidence of IBD greatly varied based on the geographical region. The regions with the highest IBD burden were Europe (0.2-23/100000) and North America (1.1-15.2/100000). The regions with the lowest reported IBD incidence were Oceania (2.9-7.2/100000), Asia (0.5-11.4/100000), Latin America (0.2-2.4/100000) and Africa (0.0-0.9/100000). The regions with the highest reported incidence of CD were North America (13.9/100000) and Europe (12.9/100000), while the highest rates of UC were 15.0/100000 in Europe and 10.6/100000 in North America. The highest incidence of IBD-U was 3.6/100000 in Europe and 2.1/100000 in North America. The current global status of the IBD incidence is shown in Figures 2-5.

Time trends in pediatric IBD

The incidence of IBD has been increasing worldwide. The time-trend analysis illustrated an increasing or stable incidence in North America, Europe and Oceania and an increasing incidence in newly industrialized countries in Asia, the Middle East and Africa. Of the 41 articles reporting the statistical significance ($P < 0.05$)
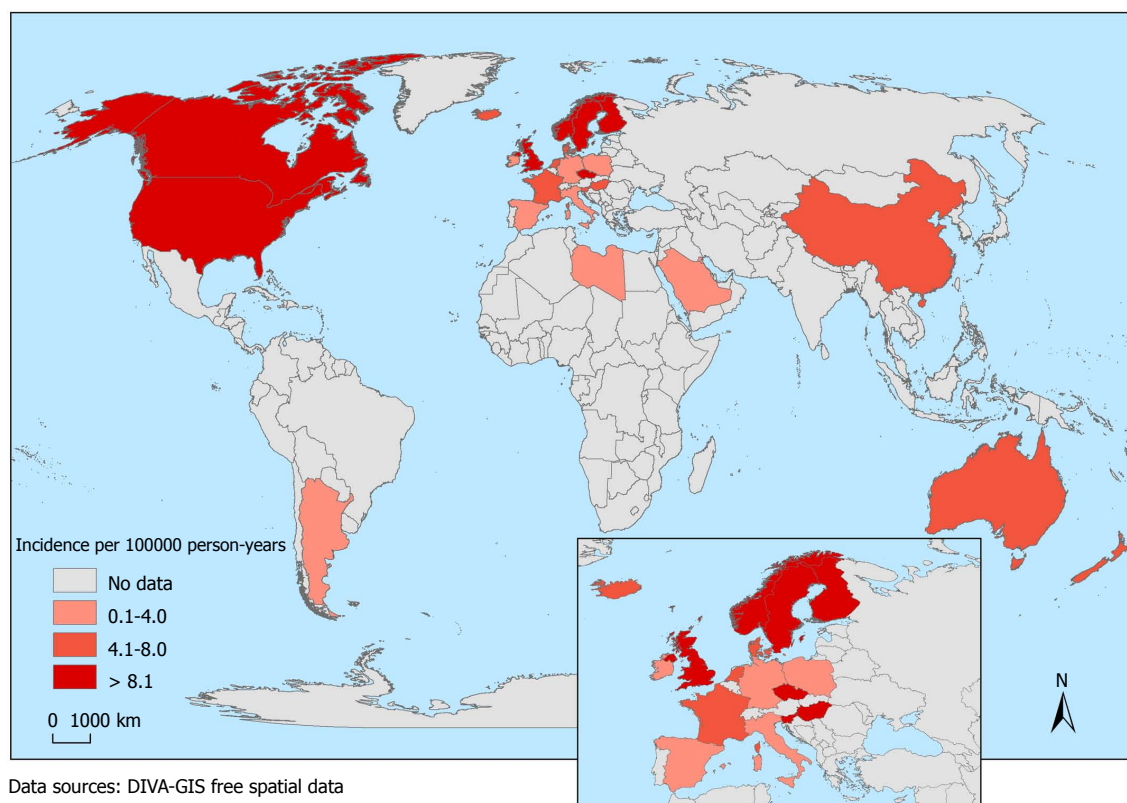
Figure 2 Worldwide pediatric inflammatory bowel disease incidence rates. Inflammatory bowel disease (IBD) choropleth map of global incidence of pediatric IBD divided into four colors representing unknown, low, indeterminate and high occurrence of disease. Grey reflects absence of data.

of a time-trend analysis of the overall IBD incidence, 30 (73%) studies reported an increasing incidence, 11 (27 %) studies reported no significant changes, and no studies reported a decreasing incidence. Of the 71 studies that calculated the CD incidence, 48 (67%) studies reported a significant increase, 2 (3%) studies reported a significant decrease, and 21 (30%) studies observed no significant changes. Of the 63 UC studies, 29 (46%) studies reported a significant increase, 29 (46 %) studies reported no significant changes, and 5 (8%) studies reported a significant decreasing trend. Of the 19 studies calculating the IBD-U incidence, 2 (11%) studies reported a significant increase, and 17 (89%) studies reported no significant change.

Description of incidence studies involving migrant and racial groups

Ten studies stratified according to migrant and race/ethnicity in South Asian (SA), Asian, African-American, Hispanic, Caucasian and Polynesian populations[16-26]. The two studies performed in Canada described the SA pediatric population, and one study compared non-immigrants to SA immigrants[18,25]. The three UK studies described the incidence in children with an Asian background[16,17,27]. The four studies performed in the US investigated Hispanic, Asian, African-American and Caucasian populations[20-22,28]. One study performed in New Zealand (NZ) described Polynesian children[26], one study performed in Australia compared Middle

Eastern ethnicities[24], one study described the Faroese Islands[19], and the same authors described the risk of IBD in first-generation Faroese immigrants.

DISCUSSION

This rigorous and timely review has several important contributions. First, wide geographical and temporal variations were observed globally. Second, North America, Northern Europe and the UK have the highest incidence worldwide. The incidences of IBD in Southern and Eastern Europe and the Southern Hemisphere also appeared to be high, whereas the incidence was lower, but climbing, in Africa, South America, and Asia. Third, the incidence of IBD is substantially increasing in worldwide regions; however, data published during the previous two decades demonstrate the plateauing incidence of IBD in the Western world after a previously documented increase[22,29-38], but incidence remains high. Currently, the incidence might be sharply increasing in Southern and Eastern Europe and in Oceania. IBD is emerging in other parts of the world (*i.e.*, certain parts of Asia/the Middle East, Africa) approaching the rates reported in westernized nations, but given the accelerating incidence found in many of these areas, the incidence is expected to increase**.** These trends clearly parallel those of the West that occurred along with the increasing development more than four decades ago indicating an emerging epidemic of IBD

Figure 3 Worldwide pediatric Crohn's disease incidence rate. Crohn's disease choropleth map of global incidence of pediatric inflammatory bowel disease divided into four colors representing unknown, low, indeterminate and high occurrence of disease. Grey reflects absence of data.

worldwide. This gap is considerably less pronounced in 2018, and the narrowing differential gap may have important implications for the worldwide IBD sequelae. The incidence has increased in recent decades up to 23 for IBD in Finland, 13.9 for CD in Canada, 15 for UC in Finland, and 3.6/100000 for IBD-U in the Netherlands. Few studies using north-south/west-east gradients have demonstrated particularly high rates in the north[39-42], except for northern France[43] and Spain[44]. Although our review did not specifically investigate disease incidence gradients, this phenomenon has been less prominent over the last three decades[45-51].

The global incidence rates of IBD have risen during the 20th century but data obtained during the previous two decades are conflicting. The incidence of IBD has increased mainly due to pediatric CD[42,51,52], whereas the incidence of UC has remained stable, although an inverse distribution of CD and UC has been reported[2,4,20,31,32,38,53-57]. Since 1950, 60% of CD and 20% of UC pediatric studies have shown a significant increase in incidence[4]. 75% of CD and 60% of UC studies show increasing incidence in adults[55]. In our systematic review, since 1985, 67% of studies investigating CD, 46% of studies investigating UC, and 11 % of studies investigating IBD-U have reported significant increasing in incidence worldwide. Thus, the incidence rates might be increasing in virtually all regions worldwide. However, the increase in UC was more modest. We intriguingly suggest that the rising

incidence of UC may be attributable to the fact that in the emerging areas with a low incidence of IBD, UC has emerged first, followed by CD after a variable period[58], similar to trends in earlier studies from the West[4,56,59-63].

CD predominates over UC and IBD-U in areas with a high IBD incidence. Recent data indicate higher rates of pediatric CD than UC in Europe and North America, except in Scandinavia[64-66], Northern California[20], Southern[63] and Eastern Europe[67], where the incidence of UC exceeds that of CD. The reasons for these striking differences in the rates among the three subtypes of IBD remain uncertain[28,68,69]. IBD-U is more frequently found in children than adults (children 12.7% *vs* adults 6.0%, *P* < 0.0001)[70].

Considerable geographical diversity in IBD is observed. Moreover, several studies have reported different incidence rates within a country[31,65], high-lighting the role of the environment[25]. Global data exploring the similarities and differences might call for future studies to extensively study genetic-environment interactions[71] providing an opportunity to further identify the contributing factors in locations where IBD is emerging rapidly[72]. Rates among ethnical/racial groups raise further questions[7] regarding why the children of immigrants from the developing world have increased rates of IBD. For example, the incidences of IBD among immigrants of SA origin in Canada[18,25], the UK[16,27], and in immigrants of Middle-Eastern descent in Australia have been reported[24]. In the state of

Figure 4 Worldwide pediatric ulcerative colitis incidence rate. Ulcerative colitis choropleth map of global incidence of pediatric inflammatory bowel disease divided into four colors representing unknown, low, indeterminate and high occurrence of disease. Grey reflects absence of data.

Georgia, the highest incidence of CD in African American children was reported[23]. The key factor of migration influencing disease onset is likely exposure to a different environment than that in the country of origin[7,73]. Additionally, indigenous populations in developed countries have a much lower IBD incidence[74].

North America

The incidence of IBD in North America is among the highest in the world and is increasing[4,20,22]. There are no national cohorts of pediatric IBD in the USA because the health system is not ideal for conducting population studies[75]. In general, data have been obtained from single regions and must be extrapolated to other geographical regions of the United States. The incidence of IBD in the entire state of Wisconsin (2000-2007) was 9.5/100000[22], which is similar to the incidence reported in Ontario, Canada[54], but the incidence of both CD and UC has remained stable. Regarding the members of the Kaiser Permanente Northern California health plan (KPNC) (1996-2006), the data somewhat differed likely due to the different mix of races/ethnicities than that observed in other parts of the United States. The incidence of UC demonstrated a significant 2.7-fold increase, and CD remained stable[20]. Hispanic and SA children had predominantly UC, suggesting the presence of possible etiological differences among ethnicities. Another study conducted in KPNC reported similar levels of IBD[76], while the incidence in children

of African-American origin in Georgia was much higher (7.1/100000)[23]. In a Texas cohort, an increasing incidence of IBD among children with evidence of more CD than UC and IBD-U from 1991 to 2002 has been reported. Caucasians had a higher IBD incidence rate than African-Americans or Hispanics, and African Americans had predominantly CD[52]. Comparable values have been reported in Wisconsin. The mean incidence of CD was double that of UC. An equal IBD incidence was observed among all ethnic groups[21]. In the Olmsted County population, the incidence was 4.8 for CD and 3.2/100000 for UC, and remained stable between 1990 and 2000[36,58]. Data from Texas, Georgia, Wisconsin and from comparable studies in KPNC found rates similar to those in Olmsted County[20,76]. However, the ratio of CD/UC cases was greater in Olmsted County, Atlanta and Wisconsin, compared to the greater incidence of UC in KPNC. These results may be attributable to the ethnic demographics of the respective populations. In contrast, a much higher rat was found in the state of Rhode Island[77] compared with older cohorts from other parts of the U.S.[21,23,36,52].

The incidence of IBD in Canada is among the highest reported to date as documented in previous single-province studies[18,78-82] and large multi-province trials[31,39,83]. The incidence in Ontario has steadily increased from 9.5 (in 1994) to 11.4/100000 (in 2005). However, the incidence of UC was stable[54], and the incidence significantly increased from 9.4 to 13.2/100000

Figure 5 Worldwide pediatric inflammatory bowel disease-unclassified incidence rate. Inflammatory bowel disease-unclassified choropleth map of global incidence of pediatric inflammatory bowel disease divided into four colors representing unknown, low, indeterminate and high occurrence of disease. Grey reflects absence of data.

(1994-2009)[84]. Although the incidence of IBD in Ontario decreased from 14.3 to 12.4/100000 in 1997-2006, the incidence of CD nearly doubled[81], which is similar to another Canadian study[39]. Among the best studies in the region, 3 studies determined the incidence of IBD in five provinces of Canada[31,83,84]. The overall incidence was 9.7/100000, and CD was the predominant form of IBD similar to data from Nordic countries[85]. The incidence of IBD was the lowest in Manitoba and the highest in Nova Scotia. The incidence of IBD remained stable after stratifying into CD and UC during the first decade of the twenty-first century, except for an increase in incidence among the youngest group. By extrapolating the results to the entire country, approximately 650 children are diagnosed with IBD yearly, affecting up to 2695 children (< 16 years) in 2008[31]. Similarly, a study from Ontario observed the most rapid increase in children (< 10 years) between 1994 and 2008[86]. Recent data from Canada suggest that the rate of incidence of pediatric IBD is plateauing, indicating a reversal after a long period of ongoing increase[18,31,78,83].

Latin America

In Argentina, IBD remains uncommon according to a study conducted from 2012 to 2013[87]. The total incidence was 0.4/100000 ranging from 0.2 to 2.4/100000. We could not find other data related to Argentinian children or children in other countries in Latin America for comparison.

Northern and Western Europe

The incidence rates reported in Scandinavia are among the highest rates published to date indicating higher rates of CD than UC and IBD-U but Finland is among the few countries that reportedly show a predominance of UC, whereas the incidence of CD has become relatively greater than that of UC in North America and the UK[20,64,66]. The incidence of IBD has almost doubled between 1987 and 2003[64] and tripled between 1987 and 2014, with a steeper increase in the incidence of UC compared to that of CD[66]. The incidence of UC increased from 4 to 9/100000 and that of CD increased from 2 to 5/100000[65,88-90] confirming a strongly increasing trend in Finland since the late 1980s. A Norwegian study covered the period of 1993-2004[60]. The incidence of IBD did not change and a trend towards an increase in CD and reduction in UC was recorded, which is similar to the finding of the IBSEN study of 1990-1994[91]. A subsequent study (2005-2007) performed in the same catchment area[92] showed that the incidence of IBD was 10.6/100000 indicating a marked increase in the incidence of CD although incidence of UC has been stabilized in Southeastern Norway compared with the rates over the previous 15 years[60,93-96]. Similarly, Canada had high a incidence of 9.7/100000[31]. The incidence of CD between 1984 and 1985 was 2.5/100000, whereas the incidence of UC was 4.3/100000 with a lower incidence of UC in Western

Norway compared to that in Southeastern Norway[97], which is similar to the results reported in a subsequent study in Southeastern Norway (1990-1994)[85]. An increase in the incidence of CD was observed in Northern Stockholm (1990-2001), while the incidence of UC was stable, and a significant increase was observed in the overall incidence of IBD[59]. In a follow-up paper (2002-2007), the incidence of IBD had plateaued. The incidence rate was 9.2 for CD and 2.8/100000 for UC[38] and the incidence of UC significantly increased but not of CD. The incidence of IBD significantly increased between 2000 and 2007 compared to that reported in an earlier study conducted in the same region (1990-2001)[38]. These rates are relatively higher than those reported in other studies on pediatric IBD, although the rates are similar to those reported in studies conducted in Canada[54,81], Norway[92], and Finland[65]. Lindberg *et al*[68] suggested that the incidence of UC increased (from 1.4 to 3.2/100000), whereas that of CD and IBD-U remained stable (1984-1995), which is similar to the results of a study conducted from 1963-1987[98]. The incidence of IBD, CD, UC and IBD-U was 6.9, 3.8, 2.1, and 1.1/100000, respectively, in Northern Stockholm (1990-1998). After more than a decade of a stable incidence of IBD in Scandinavia[35], the incidence of CD significantly increased from 1990 to 1998, while the corresponding incidence of UC and IBD-U remained unchanged[99]. As a part of the Swedish ICURE study (2005-2009), the incidence of pediatric IBD in Uppsala County, just north of Stockholm, was among the highest reported in Europe[100,101]. In Denmark, the incidence of IBD has steadily increased from the 1960s until 2013[102-104], except for in one study[105]. An increase in IBD from the 1980s to 2013 was observed, but the incidence rates increased the most in patients (< 15 years) with CD[103]. The incidence rate of UC increased from 1962 to 1987 in the county of Copenhagen[106], and Fonager *et al*[107] discovered an increasing incidence of CD but a rather stable incidence of UC with a tendency towards decreasing from 1987 to 1992. Compared to earlier Danish investigations, the incidence of CD had increased nearly 15-fold, whereas the incidence of UC remained stable between 1962 and 2006[34,102,108]. Another study observed an insignificant increase in the incidence of IBD between 1998 and 2004[33], indicating that the previously observed increasing incidence might be levelling. A significant increase in the incidence of IBD was also reported in recent Danish nationwide comparisons from 1995 to 2013 [109-111]. The IBD incidence (< 19 years) has increased in isolated regions as the Faroe Islands (part of the Danish realm) (1960-2014[19] with the predominance of UC comparable to findings obtained in the Nordic countries[64,66,105]. The incidence among Icelandic children is closest to that observed in Denmark[34] and Sweden[59] but lower than that observed in Norway[54,92]. Between 1980 and 2010, a sharp increase in IBD incidence was observed, however, the incidence levelled from 2000 to 2010[29,112] similar to

the findings in Denmark and Wisconsin[22,34] but lower compared to other Northern countries.

Sawczenko *et al*[16] discovered an incidence of 5.3/100000 in the British Isles; CD was twice as common as UC, accounting for approximately 700 new cases/year in the UK and the Republic of Ireland. A greater proportion of SA children had UC than non-immigrants[16]. In Scotland, incidence data spanning over 40 years showed a dramatic increase in IBD with a marginal decrease in the incidence of UC but an increasing incidence of CD from 1968 to 1983. In follow-up studies, the increase in CD continued between 1990 and 1992[61,113]. In contrast, the incidence of CD continues to increase, and the incidence of UC is also apparently increasing from 1981 to 1995[53]. Other Scottish studies showed an increase in the incidence of CD from 1981 to 1995 but no difference was observed in the incidence of UC[16,114,115]. By comparing the periods 1990-1995 to 2003-2008, significant increases were observed in the incidence of IBD, CD and UC[2]. Data obtained in Wessex, England reported an incidence of 9.37/100000 which significantly increased from 2002 to 2012[116]. The most recent figures (2013-2017) were reported and compared to previously published Wessex data, demonstrating the most contemporary incidence and trend over 16 years[117]. Similar findings have been observed among Welsh children[57] and appears to have plateaued between 1995 and 1997[118]. In 1995-2003, the overall incidence of IBD was 5.4/100000 and had reached a plateau[30,118], but data obtained during 1996-2005 show that the incidence of CD is continuing to slowly increase in Cardiff[119]. An increasing incidence of IBD in Irish children was observed between 1998 and 2014[16,120,121], which is consistent with the global trends[2,4]. Another study also confirms the continuous increase in the incidence of IBD, particularly UC[121].

In Austria, the overall incidence of CD and UC have increased from 1997 to 2007[122]. This finding is in contrast to Germany[123] and the Netherlands[124]. For example, a German study did not show any significant change in the IBD incidence[123]. Worldwide, strikingly, the high proportion of IBD-U was observed in the Netherlands[124] and the incidence of IBD cases is comparable with that reported in other European countries. In France, the incidence of CD significantly increased, while the incidence of UC remains unchanged from 1988 to 2011[32,43,125-128]. The most remarkable observation (the EPIMAD Registry) has been a striking increase from 1988 to 2008 in the incidence of CD (< 19 years)[125], and the rates also significantly rose from 1988 to 2011[32,126,127] Surprisingly, in Corsica, using the same registry (EPIMAD), the incidence of CD was close to that observed in other metropolitan French regions; however, the incidence of IBD for UC in Corsica is two-fold higher than that reported in other French regions[128]. In Brittany, the incidence of IBD in childhood was similar to data obtained in Northern France and Nord-Pas-de-Calais[129,130]. Between 1988 and 2011, a dramatic increase was observed in the incidence of both

UC and CD in French adolescents[131]. Other than the Swiss IBD Cohort Study (SIBDCS)[132] and the Belgian registry for pediatric CD (BELCRO)[133], up-to-date data regarding the incidence and trends are lacking.

Southern Europe
A registry in Italy (1996-2003) showed a significant increase in the incidence of IBD from 0.89 to 1.39/100000 in all 3 pathologies[63], which is comparable to the incidence in Lombardia (1990-1993)[134]. Of note, Italy had a low incidence of IBD earlier, with an initial increase in UC exceeding CD and IBD-U, followed by an increase in the CD incidence, while the UC incidence was stable from 1998-2003[63]. In contrast, the incidence of IBD in central Italy (1978-1992) was comparable to that in the Nordic countries[135,136]. The incidence of IBD was shown to have significantly increased in Spain[44,137]. The SPIRIT (1996-2009) and EXPERIENCE registries (1985-1995) contribute to the complete description of the changes in pediatric IBD in Spain. A three-fold collective increase in IBD (1996-2009) was observed, with another three-fold increase in CD and a two-fold increase in UC, while a lower proportion of IBD-U was described[42]. According to the two latter studies extending the trends to a full 25-year period, these registries showed a sixteen-fold increase in Spain[42,51]. Notably, the incidence of IBD (mainly CD) (18.3/100000) in the Vigo area was the highest compared to that in former Spanish pediatric cohorts[50]. In Malta, UC showed an almost significant increasing trend, but no significant trend in CD was observed[138].

Central and Eastern Europe
A sharp increase in the incidence of IBD is particularly noticeable in the Czech Republic, Hungary, Slovenia and Croatia, but not in Poland. The results of the 3 studies conducted in the Czech Republic are comparable to the West. Pozler *et al*[139] showed a five-fold increase in the incidence of CD. Kolek *et al*[140] published results from Moravia (the eastern part of the Czech Republic) showing increasing incidence of CD and UC between 1999 and 2001. The Czech Republic has among the highest rates of IBD worldwide as recently observed by our group (2000-2015)[45] (10.0, 6.2, 2.8 and 1.0/100000 for IBD, CD, UC and IBD-U, respectively) and have been shown to be increasing in future projections[45]. In neighboring Poland, UC incidence was higher than that of CD with significant regional differences, but the incidence was markedly lower than that observed in the West. Of note the incidence of IBD-U was surprisingly high[67]. An increasing incidence of IBD has been reported in Hungary[46,141,142] comparable to the rate of Slovenia from 1994 to 2010[48,49,143]. One other publication[47] reported much higher rates in Croatia (2000-2004) compared to previous reports[144,145].

Africa
Expectedly, knowledge regarding the incidence of IBD in the entire African pediatric population is limited, but the incidence of IBD increased from 0.0/100000 in 1997 to 0.2/100000 in 2002 and 0.9/100000 in 2006 in Libya[146].

Asia/the Middle East
In Israel, the estimated incidence of CD and UC was 3.7 and 0.9/100000, respectively[147,148], which are comparable, but at the lower end, with those in the West[16,21]. 2 studies reported that the IBD incidence (0.5/100000) is lower than that in Western countries among children in Saudi Arabia from 1993 to 2012[149,150]. A higher incidence was reported in Kuwait and Bahrain (states neighboring Saudi Arabia). In Kuwait, the incidence was more than triple that reported in neighboring Saudi Arabia[151]. This finding provides an annual incidence of 2.16/100000 for IBD, whereas the incidence of CD is 1.53, UC is 0.6 and IBD-U is 0.03/100000. A remarkable finding reported in the Arabian Gulf region is the high incidence of CD in Bahrain[152], which is comparable to Western areas. The lower incidence of IBD in Asia compared with that in Western countries is not universal, and the incidence in China is higher than that in other regions in Asia. This incidence is considered low compared to that in North America and the Nordic countries but not that low compared to the incidence in Scotland[53,113,114] and France[43] and is higher than that in Italy[63]. In China, a multicenter audit of over a decade of experience with childhood IBD between 2000 and 2010 in Shanghai has shown a steadily increasing trend (< 14 years). The incidence of IBD in 2010 was 6.1/100000, which is 12-fold higher than the incidence in 2001[153]. Recent data on Taiwan also demonstrated a substantial increase in the incidence of IBD, which is mainly attributable to CD, while the incidence of UC did not change significantly[154]. Singapore also witnessed a remarkable increase from 2000-2008; the incidence rates were 5.2-fold greater than those assessed 9 years earlier (from 2.2 to 11.4/100000)[155], which is similar to a report from Scandinavia[66]. The incidence of IBD has been increasing in Korea recently[156,157].

Australasia
Early studies conducted in Australia and NZ (collectively termed Australasia) mirror the incidence observed in the Northern Hemisphere[54,65]. Two Victorian studies from the same area of Australia clearly show increasing rates of both CD and UC. The incidence of CD increased 10-fold over 30 years until 2001[158]. Additionally, an eleven-fold increase was seen in UC with particular increases in the early 1990s, and the incidence has yet to plateau[159]. Using the 1983-1998 population data, the incidence was estimated to be 2.01/100000 for CD among Victorian children with a documented increase[37]. A study conducted in the Sydney area showed much higher rates of IBD in children of Middle-Eastern descent[24]. In contrast, low rates of IBD were

observed in the following indigenous populations: Aborigines and Maori[74]. Yap *et al*[26] calculated the incidence of IBD, CD and UC to be 2.9, 1.9 and 0.5/100000, respectively in NZ, which is at the lower end compared with the incidence in Europe. Recently, Lopez *et al*[160] provided important data pertaining to the incidence of pediatric IBD in NZ. The incidence of IBD, CD, UC and IBD-U in NZ in 2015 were 5.2, 3.5, 1.0 and 0.7/100000, respectively. A 4-fold increase was observed in the incidence of childhood IBD in the Canterbury Province of NZ between 1996 and 2015. The annual incidence rate was 7.18/100000 with the preponderance of CD over UC (8.4:1)[160,161].

Limitations of the study

Limitations include heterogeneity in population characteristics among the different studies which were also conducted at different times. Most countries lack accurate estimates of the incidence of pediatric IBD. A direct inter-region comparison may be limited due to the use of different diagnostic criteria and geographic distribution[25]. Also, study quality, case ascertainment, different database capture systems, and methodological problems demonstrated heterogeneity, emphasizing the importance of nationwide registries for retrieval of specific health data[62] including a well-established referral system with uniform criteria[40]. This recommendation underlines the need for uniform diagnostic guidelines for the accuracy of comparison among populations[38,162]. Some studies observed crude incidence rates, while other studies reported age- and/ or sex-adjusted rates. Some studies did not reflect the countries' true incidence, because selected areas of the country were sampled instead of the entire country. We should target large databases at the national/ international levels providing a more comprehensive analysis[41]. The other limitation rests with the retrospective design of a number of studies and a better categorisation of migrants. The differences in the various age brackets with great impact on incidence rate should be considered as studies involving a higher defined age limit had higher incidence figures[92]. Despite these limitations, these considerations are unlikely to have had major effects on the reporting the changes in the incidence of IBD across time and geography.

Conclusion

The incidence of pediatric IBD (mainly CD) has recently dramatically increased emerging as a globally important changing pediatric disease. In a rapidly changing world, the dramatically increasing incidence of IBD has been observed in the Western world, Oceania and Eastern and Southern Europe, despite a stabilization of incidence rates in the West. The incidence appears to be rising both in newly industrialized and developing countries, and among first-generation of immigrants. Regarding IBD, knowledge of its etiology is limited, and awareness of the patterns of its global incidence could offer

new clues, but the complex interplay of genetic and environmental factors remains unclear. Investigations of IBD performed where it is rapidly emerging provide an opportunity to identify the contributing factors. Efforts at global co-ordination for more prospective, population-based studies in children should be encouraged.

ARTICLE HIGHLIGHTS

Research background
The incidence of inflammatory bowel disease (IBD) is increasing globally. Multiple studies have reported the pediatric IBD incidence in individuals over the past few years. However, the global and regional IBD incidences in childhood and their trends over time are not well reported. The highest pediatric incidence is traditionally observed in industrialized countries in North America and Western Europe. The incidence of IBD is increasing in both developed and developing countries. The variations in the disease incidence may reflect differences in the distribution of various environmental triggers for the disease within specific areas. The changing incidence of pediatric IBD worldwide provides an opportunity to study disease etiology.

Research motivation
The incidence of IBD is increasing worldwide in both adults and children. Thus, epidemiological knowledge is essential for defining new etiological hypotheses and predictors of the development of IBD to better define how environmental factors might influence disease onset and to guide future studies. Furthermore, additional evidence has recently become available due to the publication of previous reviews, but many incidence rates have since changed. Consequently, currently, a window of opportunity exists for the completion of a new, rigorous pediatric systematic review.

Research objectives
The authors aimed to summarize up-to-date studies investigating the incidence of pediatric-onset IBD and track the changes over time based on a comprehensive search of credible published pediatric studies and current knowledge regarding pediatric IBD incidence.

Research methods
A systematic review was performed using Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. We searched electronic databases (MEDLINE, EMBASE, and Cochrane Library). Studies investigating the incidence and trends of pediatric IBD over time were eligible for inclusion. Interactive maps and temporal trends were used to illustrate the incidences of and changes in IBD.

Research results
One hundred forty studies met the inclusion criteria, demonstrating a substantial increase in the incidence of pediatric IBD and great geographic variations. The incidence of IBD remains the highest in the northern populations of Europe and America and has remained stable or even decreased. Rising rates of pediatric IBD were observed in previously low-incidence areas and much of the developing world, and among children of immigrants. The incidence rates of Crohn's disease (CD) and ulcerative colitis (UC) vary worldwide between 0.2/100000 and 13.9/100000 and between 0.1/100000 and 15/100000, respectively. In the time-trend analyses, 67% of CD and 46% of UC studies reported a significant increase.

Research conclusions
This study is among the most comprehensive studies to summarize the global IBD incidence. The IBD incidence is increasing or stable over time in both developed and developing regions of the world, indicating an emerging epidemic of the disease outside the Western world, whereas those in Northern Europe may have reached a plateau. The reasons contributing to these continued increases remain unclear. Whether genetic or environmental factors are the cause of these differences remains to be determined. Investigations of

IBD performed in locations where it is emerging rapidly provide an opportunity to further identify the causative factors within specific populations. Whether the incidence of IBD in children will continue to increase or remain static is unclear.

Research perspectives

Our data may serve as an essential resource for future studies and can be used to prioritize public health efforts in areas with the highest incidence. Knowing the increasing incidence of IBD and different geographic distribution may provide new insight into the etiology of pediatric IBD and direct future investigative studies. We must find ways to match genetic and environmental factors to pediatric IBD. An understanding of the early evolution of IBD is important and must be further investigated to unravel its etiology. This understanding is particularly important for preventing or curing the disease during the early stages. Attempts to perform studies with global coordination and additional prospective, population-based studies should be encouraged.

REFERENCES

1   **Abraham BP**, Mehta S, El-Serag HB. Natural history of pediatric-onset inflammatory bowel disease: a systematic review. *J Clin Gastroenterol* 2012; **46**: 581-589 [PMID: 22772738 DOI: 10.1097/MCG.0b013e318247c32f]

2   **Henderson P**, Rogers P, Gillett P, Wilson D. The epidemiology and natural history of paediatric inflammatory bowel disease in a UK region: a prospective 14-year study. *Arch Dis Child* 2012; **97**: A53.2-A54 [DOI: 10.1136/archdischild-2012-301885.130]

3   **Ng SC**, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, Panaccione R, Ghosh S, Wu JCY, Chan FKL, Sung JJY, Kaplan GG. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet* 2018; **390**: 2769-2778 [PMID: 29050646 DOI: 10.1016/S0140-6736(17)32448-0]

4   **Benchimol EI**, Fortinsky KJ, Gozdyra P, Van den Heuvel M, Van Limbergen J, Griffiths AM. Epidemiology of pediatric inflammatory bowel disease: a systematic review of international trends. *Inflamm Bowel Dis* 2011; **17**: 423-439 [PMID: 20564651 DOI: 10.1002/ibd.21349]

5   **da Silva BC**, Lyra AC, Rocha R, Santana GO. Epidemiology, demographic characteristics and prognostic predictors of ulcerative colitis. *World J Gastroenterol* 2014; **20**: 9458-9467 [PMID: 25071340 DOI: 10.3748/wjg.v20.i28.9458]

6   **Van Limbergen J**, Russell RK, Drummond HE, Aldhous MC, Round NK, Nimmo ER, Smith L, Gillett PM, McGrogan P, Weaver LT, Bisset WM, Mahdi G, Arnott ID, Satsangi J, Wilson DC. Definition of phenotypic characteristics of childhood-onset inflammatory bowel disease. *Gastroenterology* 2008; **135**: 1114-1122 [PMID: 18725221 DOI: 10.1053/j.gastro.2008.06.081]

7   **Misra R**, Faiz O, Munkholm P, Burisch J, Arebi N. Epidemiology of inflammatory bowel disease in racial and ethnic migrant groups. *World J Gastroenterol* 2018; **24**: 424-437 [PMID: 29391765 DOI: 10.3748/wjg.v24.i3.424]

8   **Girardelli M**, Basaldella F, Paolera SD, Vuch J, Tommasini A, Martelossi S, Crovella S, Bianco AM. Genetic profile of patients with early onset inflammatory bowel disease. *Gene* 2018; **645**: 18-29 [PMID: 29248579 DOI: 10.1016/j.gene.2017.12.029]

9   **Jakobsen C**, Cleynen I, Andersen PS, Vermeire S, Munkholm P, Paerregaard A, Wewer V. Genetic susceptibility and genotype-phenotype association in 588 Danish children with inflammatory bowel disease. *J Crohns Colitis* 2014; **8**: 678-685 [PMID: 24394805 DOI: 10.1016/j.crohns.2013.12.010]

10 **Fang YH**, Luo YY, Yu JD, Lou JG, Chen J. Phenotypic and genotypic characterization of inflammatory bowel disease in children under six years of age in China. *World J Gastroenterol* 2018; **24**: 1035-1045 [PMID: 29531467 DOI: 10.3748/wjg.v24.i9.1035]

11 **von Allmen D**. Pediatric Crohn's Disease. *Clin Colon Rectal Surg* 2018; **31**: 80-88 [PMID: 29487490 DOI: 10.1055/s-0037-1609022]

12 **Liberati A**, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JP, Clarke M, Devereaux PJ, Kleijnen J, Moher D. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. *PLoS Med* 2009; **6**: e1000100 [PMID: 19621070 DOI: 10.1371/journal.pmed.1000100]

13 **United Nations.** Composition of macro Geographical (Continental) Regions, Geographical Sub-Regions, and Selected Economic and Other Groupings. Available from: URL: http://unstats.un.org/unsd/methods/m49/m49regin.htm

14 **Lennard-Jones JE**. Classification of inflammatory bowel disease. *Scand J Gastroenterol Suppl* 1989; **170**: 2-6; discussion 16-19 [PMID: 2617184 DOI: 10.3109/00365528909091339]

15 **Sanderson S**, Tatt ID, Higgins JP. Tools for assessing quality and susceptibility to bias in observational studies in epidemiology: a systematic review and annotated bibliography. *Int J Epidemiol* 2007; **36**: 666-676 [PMID: 17470488 DOI: 10.1093/ije/dym018]

16 **Sawczenko A**, Sandhu BK, Logan RF, Jenkins H, Taylor CJ, Mian S, Lynn R. Prospective survey of childhood inflammatory bowel disease in the British Isles. *Lancet* 2001; **357**: 1093-1094 [PMID: 11297962 DOI: 10.1016/S0140-6736(00)04309-9]

17 **Tsironi E**, Feakins RM, Probert CS, Rampton DS, Phil D. Incidence of inflammatory bowel disease is rising and abdominal tuberculosis is falling in Bangladeshis in East London, United Kingdom. *Am J Gastroenterol* 2004; **99**: 1749-1755 [PMID: 15330914 DOI: 10.1111/j.1572-0241.2004.30445.x]

18 **Pinsk V**, Lemberg DA, Grewal K, Barker CC, Schreiber RA, Jacobson K. Inflammatory bowel disease in the South Asian pediatric population of British Columbia. *Am J Gastroenterol* 2007; **102**: 1077-1083 [PMID: 17378907 DOI: 10.1111/j.1572-0241.2007.01124.x]

19 **Hammer T**, Nielsen KR, Munkholm P, Burisch J, Lynge E. The Faroese IBD Study: Incidence of Inflammatory Bowel Diseases Across 54 Years of Population-based Data. *J Crohns Colitis* 2016; **10**: 934-942 [PMID: 26933031 DOI: 10.1093/ecco-jcc/jjw050]

20 **Abramson O**, Durant M, Mow W, Finley A, Kodali P, Wong A, Tavares V, McCroskey E, Liu L, Lewis JD, Allison JE, Flowers N, Hutfless S, Velayos FS, Perry GS, Cannon R, Herrinton LJ. Incidence, prevalence, and time trends of pediatric inflammatory bowel disease in Northern California, 1996 to 2006. *J Pediatr* 2010; **157**: 233-239.e1 [PMID: 20400099 DOI: 10.1016/j.jpeds.2010.02.024]

21 **Kugathasan S**, Judd RH, Hoffmann RG, Heikenen J, Telega G, Khan F, Weisdorf-Schindele S, San Pablo W Jr, Perrault J, Park R, Yaffe M, Brown C, Rivera-Bennett MT, Halabi I, Martinez A, Blank E, Werlin SL, Rudolph CD, Binion DG; Wisconsin Pediatric Inflammatory Bowel Disease Alliance. Epidemiologic and clinical characteristics of children with newly diagnosed inflammatory bowel disease in Wisconsin: a statewide population-based study. *J Pediatr* 2003; **143**: 525-531 [PMID: 14571234 DOI: 10.1067/S0022-3476(03)00444-X]

22 **Adamiak T**, Walkiewicz-Jedrzejczak D, Fish D, Brown C, Tung J, Khan K, Faubion W Jr, Park R, Heikenen J, Yaffee M, Rivera-Bennett MT, Wiedkamp M, Stephens M, Noel R, Nugent M, Nebel J, Simpson P, Kappelman MD, Kugathasan S. Incidence, clinical characteristics, and natural history of pediatric IBD in Wisconsin: a population-based epidemiological study. *Inflamm Bowel Dis* 2013; **19**: 1218-1223 [PMID: 23528339 DOI: 10.1097/MIB.0b013e318280b13e]

23 **Ogunbi SO**, Ransom JA, Sullivan K, Schoen BT, Gold BD. Inflammatory bowel disease in African-American children living in Georgia. *J Pediatr* 1998; **133**: 103-107 [PMID: 9672520 DOI: 10.1016/S0022-3476(98)70187-8]

24 **Naidoo CM**, Leach ST, Day AS, Lemberg DA. Inflammatory bowel disease in children of middle eastern descent. *Int J Pediatr* 2014; **2014**: 906128 [PMID: 24987422 DOI: 10.1155/2014/906128]

25 **Benchimol EI**, Mack DR, Guttmann A, Nguyen GC, To T, Mojaverian N, Quach P, Manuel DG. Inflammatory bowel disease in immigrants to Canada and their children: a population-based cohort study. *Am J Gastroenterol* 2015; **110**: 553-563 [PMID: 25756238 DOI: 10.1038/ajg.2015.52]

26 **Yap J**, Wesley A, Mouat S, Chin S. Paediatric inflammatory bowel

disease in New Zealand. *N Z Med J* 2008; **121**: 19-34 [PMID: 18841182]

27   **Probert CS**, Jayanthi V, Pinder D, Wicks AC, Mayberry JF. Epidemiological study of ulcerative proctocolitis in Indian migrants and the indigenous population of Leicestershire. *Gut* 1992; **33**: 687-693 [PMID: 1307684 DOI: 10.1136/gut.33.5.687]

28   **Malaty HM**, Mehta S, Abraham B, Garnett EA, Ferry GD. The natural course of inflammatory bowel disease-indeterminate from childhood to adulthood: within a 25 year period. *Clin Exp Gastroenterol* 2013; **6**: 115-121 [PMID: 23901288 DOI: 10.2147/CEG.S44700]

29   **Agnarsson** U, Björnsson S, Jóhansson JH, Sigurdsson L. Inflammatory bowel disease in Icelandic children 1951-2010. Population-based study involving one nation over six decades. *Scand J Gastroenterol* 2013; **48**: 1399-1404 [PMID: 24164345 DOI: 10.3109/00365521.2013.845799]

30   **Ahmed M**, Davies IH, Hood K, Jenkins HR. Incidence of paediatric inflammatory bowel disease in South Wales. *Arch Dis Child* 2006; **91**: 344-345 [PMID: 16551790 DOI: 10.1136/adc.2004.067413]

31   **Benchimol EI**, Bernstein CN, Bitton A, Carroll MW, Singh H, Otley AR, Vutcovici M, El-Matary W, Nguyen GC, Griffiths AM, Mack DR, Jacobson K, Mojaverian N, Tanyingoh D, Cui Y, Nugent ZJ, Coulombe J, Targownik LE, Jones JL, Leddin D, Murthy SK, Kaplan GG. Trends in Epidemiology of Pediatric Inflammatory Bowel Disease in Canada: Distributed Network Analysis of Multiple Population-Based Provincial Health Administrative Databases. *Am J Gastroenterol* 2017; **112**: 1120-1134 [PMID: 28417994 DOI: 10.1038/ajg.2017.97]

32   **Chouraki V**, Savoye G, Dauchet L, Vernier-Massouille G, Dupas JL, Merle V, Laberenne JE, Salomez JL, Lerebours E, Turck D, Cortot A, Gower-Rousseau C, Colombel JF. The changing pattern of Crohn's disease incidence in northern France: a continuing increase in the 10- to 19-year-old age bracket (1988-2007). *Aliment Pharmacol Ther* 2011; **33**: 1133-1142 [PMID: 21488915 DOI: 10.1111/j.1365-2036.2011.04628.x]

33   **Jakobsen C**, Wewer V, Urne F, Andersen J, Faerk J, Kramer I, Stagegaard B, Pilgaard B, Weile B, Paerregaard A. Incidence of ulcerative colitis and Crohn's disease in Danish children: Still rising or levelling out? *J Crohns Colitis* 2008; **2**: 152-157 [PMID: 21172205 DOI: 10.1016/j.crohns.2008.01.006]

34   **Jakobsen C**, Paerregaard A, Munkholm P, Wewer V. Paediatric inflammatory bowel disease during a 44-year period in Copenhagen County: occurrence, course and prognosis--a population-based study from the Danish Crohn Colitis Database. *Eur J Gastroenterol Hepatol* 2009; **21**: 1291-1301 [PMID: 19581873 DOI: 10.1097/MEG.0b013e32832a4ed6]

35   **Lapidus A**, Bernell O, Hellers G, Persson PG, Löfberg R. Incidence of Crohn's disease in Stockholm County 1955-1989. *Gut* 1997; **41**: 480-486 [PMID: 9391246 DOI: 10.1136/gut.41.4.480]

36   **Loftus EV Jr**, Silverstein MD, Sandborn WJ, Tremaine WJ, Harmsen WS, Zinsmeister AR. Crohn's disease in Olmsted County, Minnesota, 1940-1993: incidence, prevalence, and survival. *Gastroenterology* 1998; **114**: 1161-1168 [PMID: 9609752 DOI: 10.1016/S0016-5085(98)70421-4]

37   **Ponsonby AL**, Catto-Smith AG, Pezic A, Dupuis S, Halliday J, Cameron D, Morley R, Carlin J, Dwyer T. Association between early-life factors and risk of child-onset Crohn's disease among Victorian children born 1983-1998: a birth cohort study. *Inflamm Bowel Dis* 2009; **15**: 858-866 [PMID: 19107784 DOI: 10.1002/ibd.20842]

38   **Malmborg P**, Grahnquist L, Lindholm J, Montgomery S, Hildebrand H. Increasing incidence of paediatric inflammatory bowel disease in northern Stockholm County, 2002-2007. *J Pediatr Gastroenterol Nutr* 2013; **57**: 29-34 [PMID: 23459320 DOI: 10.1097/MPG.0b013e31828f21b4]

39   **Bernstein CN**, Wajda A, Svenson LW, MacKenzie A, Koehoorn M, Jackson M, Fedorak R, Israel D, Blanchard JF. The epidemiology of inflammatory bowel disease in Canada: a population-based study. *Am J Gastroenterol* 2006; **101**: 1559-1568 [PMID: 16863561 DOI: 10.1111/j.1572-0241.2006.00603.x]

40   **Shivananda S**, Lennard-Jones J, Logan R, Fear N, Price A, Carpenter L, van Blankenstein M. Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European Collaborative Study on Inflammatory Bowel Disease (EC-IBD). *Gut* 1996; **39**: 690-697 [PMID: 9014768 DOI: 10.1136/gut.39.5.690]

41   **Burisch J**. Crohn's disease and ulcerative colitis. Occurrence, course and prognosis during the first year of disease in a European population-based inception cohort. *Dan Med J* 2014; **61**: B4778 [PMID: 24393595]

42   **Martín-de-Carpi J**, Rodríguez A, Ramos E, Jiménez S, Martínez-Gómez MJ, Medina E; SPIRIT-IBD Working Group of Sociedad Española de Gastroenterología, Hepatología y Nutricion Pediátrica. Increasing incidence of pediatric inflammatory bowel disease in Spain (1996-2009): the SPIRIT Registry. *Inflamm Bowel Dis* 2013; **19**: 73-80 [PMID: 22535521 DOI: 10.1002/ibd.22980]

43   **Auvin S**, Molinié F, Gower-Rousseau C, Brazier F, Merle V, Grandbastien B, Marti R, Lerebours E, Dupas JL, Colombel JF, Salomez JL, Cortot A, Turck D. Incidence, clinical presentation and location at diagnosis of pediatric inflammatory bowel disease: a prospective population-based study in northern France (1988-1999). *J Pediatr Gastroenterol Nutr* 2005; **41**: 49-55 [PMID: 15990630 DOI: 10.1097/01.MPG.0000162479.74277.86]

44   **Rodrigo L**, Riestra S, Niño P, Cadahía V, Tojo R, Fuentes D, Moreno M, González Ballina E, Fernández E. A population-based study on the incidence of inflammatory bowel disease in Oviedo (Northern Spain). *Rev Esp Enferm Dig* 2004; **96**: 296-305 [PMID: 15180441 DOI: 10.4321/S1130-01082004000500002]

45   **Schwarz J**, Sýkora J, Cvalínová D, Pomahačová R, Klečková J, Kryl M, Včelák P. Inflammatory bowel disease incidence in Czech children: A regional prospective study, 2000-2015. *World J Gastroenterol* 2017; **23**: 4090-4101 [PMID: 28652662 DOI: 10.3748/wjg.v23.i22.4090]

46   **Lovasz BD**, Lakatos L, Horvath A, Pandur T, Erdelyi Z, Balogh M, Szipocs I, Vegh Z, Veres G, Müller KE, Golovics PA, Kiss LS, Mandel MD, Lakatos PL. Incidence rates and disease course of paediatric inflammatory bowel diseases in Western Hungary between 1977 and 2011. *Dig Liver Dis* 2014; **46**: 405-411 [PMID: 24495511 DOI: 10.1016/j.dld.2013.12.013]

47   **Sincić BM**, Vucelić B, Persić M, Brncić N, Erzen DJ, Radaković B, Mićović V, Stimac D. Incidence of inflammatory bowel disease in Primorsko-goranska County, Croatia, 2000-2004: A prospective population-based study. *Scand J Gastroenterol* 2006; **41**: 437-444 [PMID: 16635912 DOI: 10.1080/00365520500320094]

48   **Urlep D**, Blagus R, Orel R. Incidence Trends and Geographical Variability of Pediatric Inflammatory Bowel Disease in Slovenia: A Nationwide Study. *Biomed Res Int* 2015; **2015**: 921730 [PMID: 26688822 DOI: 10.1155/2015/921730]

49   **Urlep D**, Trop TK, Blagus R, Orel R. Incidence and phenotypic characteristics of pediatric IBD in northeastern Slovenia, 2002-2010. *J Pediatr Gastroenterol Nutr* 2014; **58**: 325-332 [PMID: 24135984 DOI: 10.1097/MPG.0000000000000207]

50   **Fernández A**, Hernández V, Martínez-Ares D, Sanromán L, de Castro ML, Pineda JR, Carmona A, González-Portela C, Salgado C, Martínez-Cadilla J, Pereira S, García-Burriel JI, Vázquez S, Rodríguez-Prada I; EpiCom Group. Incidence and phenotype at diagnosis of inflammatory bowel disease. Results in Spain of the EpiCom study. *Gastroenterol Hepatol* 2015; **38**: 534-540 [PMID: 25890448 DOI: 10.1016/j.gastrohep.2015.03.001]

51   **Martín-de-Carpi J**, Rodríguez A, Ramos E, Jiménez S, Martínez-Gómez MJ, Medina E, Navas-López VM; SPIRIT-IBD Working Group of SEGHNP (Sociedad Española de Gastroenterología Hepatología Nutrición Pediátrica). The complete picture of changing pediatric inflammatory bowel disease incidence in Spain in 25 years (1985-2009): the EXPERIENCE registry. *J Crohns Colitis* 2014; **8**: 763-769 [PMID: 24462789 DOI: 10.1016/j.crohns.2014.01.005]

52   **Malaty HM**, Fan X, Opekun AR, Thibodeaux C, Ferry GD. Rising incidence of inflammatory bowel disease among children: a 12-year study. *J Pediatr Gastroenterol Nutr* 2010; **50**: 27-31 [PMID: 19934770 DOI: 10.1097/MPG.0b013e3181b99baa]

53   **Armitage E**, Drummond HE, Wilson DC, Ghosh S. Increasing incidence of both juvenile-onset Crohn's disease and ulcerative colitis in Scotland. *Eur J Gastroenterol Hepatol* 2001; **13**: 1439-1447 [PMID: 11742192 DOI: 10.1097/00042737-200112000-00007]

54   **Benchimol EI**, Guttmann A, Griffiths AM, Rabeneck L, Mack DR, Brill H, Howard J, Guan J, To T. Increasing incidence of paediatric inflammatory bowel disease in Ontario, Canada: evidence from health administrative data. *Gut* 2009; **58**: 1490-1497 [PMID: 19651626 DOI: 10.1136/gut.2009.188383]

55   **Molodecky NA**, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 2012; **142**: 46-54.e42; quiz e30 [PMID: 22001864 DOI: 10.1053/j.gastro.2011.10.001]

56   **Ekbom A**, Helmick C, Zack M, Adami HO. The epidemiology of inflammatory bowel disease: a large, population-based study in Sweden. *Gastroenterology* 1991; **100**: 350-358 [PMID: 1985033 DOI: 10.1016/0016-5085(91)90202-V]

57   **Cosgrove M**, Al-Atia RF, Jenkins HR. The epidemiology of paediatric inflammatory bowel disease. *Arch Dis Child* 1996; **74**: 460-461 [PMID: 8669968 DOI: 10.1136/adc.74.5.460]

58   **Loftus CG**, Loftus EV Jr, Harmsen WS, Zinsmeister AR, Tremaine WJ, Melton LJ 3rd, Sandborn WJ. Update on the incidence and prevalence of Crohn's disease and ulcerative colitis in Olmsted County, Minnesota, 1940-2000. *Inflamm Bowel Dis* 2007; **13**: 254-261 [PMID: 17206702 DOI: 10.1002/ibd.20029]

59   **Hildebrand H**, Finkel Y, Grahnquist L, Lindholm J, Ekbom A, Askling J. Changing pattern of paediatric inflammatory bowel disease in northern Stockholm 1990-2001. *Gut* 2003; **52**: 1432-1434 [PMID: 12970135 DOI: 10.1136/gut.52.10.1432]

60   **Perminow G**, Frigessi A, Rydning A, Nakstad B, Vatn MH. Incidence and clinical presentation of IBD in children: comparison between prospective and retrospective data in a selected Norwegian population. *Scand J Gastroenterol* 2006; **41**: 1433-1439 [PMID: 17101574 DOI: 10.1080/00365520600789891]

61   **Barton JR**, Gillon S, Ferguson A. Incidence of inflammatory bowel disease in Scottish children between 1968 and 1983; marginal fall in ulcerative colitis, three-fold rise in Crohn's disease. *Gut* 1989; **30**: 618-622 [PMID: 2786488 DOI: 10.1136/gut.30.5.618]

62   **Munkholm P**, Langholz E, Nielsen OH, Kreiner S, Binder V. Incidence and prevalence of Crohn's disease in the county of Copenhagen, 1962-87: a sixfold increase in incidence. *Scand J Gastroenterol* 1992; **27**: 609-614 [PMID: 1641589 DOI: 10.3109/00365529209000127]

63   **Castro M**, Papadatou B, Baldassare M, Balli F, Barabino A, Barbera C, Barca S, Barera G, Bascietto F, Berni Canani R, Calacoci M, Campanozzi A, Castellucci G, Catassi C, Colombo M, Covoni MR, Cucchiara S, D'Altilia MR, De Angelis GL, De Virgilis S, Di Ciommo V, Fontana M, Guariso G, Knafelz D, Lambertini A, Licciardi S, Lionetti P, Liotta L, Lombardi G, Maestri L, Martelossi S, Mastella G, Oderda G, Perini R, Pesce F, Ravelli A, Roggero P, Romano C, Rotolo N, Rutigliano V, Scotta S, Sferlazzas C, Staiano A, Ventura A, Zaniboni MG. Inflammatory bowel disease in children and adolescents in Italy: data from the pediatric national IBD register (1996-2003). *Inflamm Bowel Dis* 2008; **14**: 1246-1252 [PMID: 18521916 DOI: 10.1002/ibd.20470]

64   **Turunen P**, Kolho KL, Auvinen A, Iltanen S, Huhtala H, Ashorn M. Incidence of inflammatory bowel disease in Finnish children, 1987-2003. *Inflamm Bowel Dis* 2006; **12**: 677-683 [PMID: 16917221 DOI: 10.1097/00054725-200608000-00002]

65   **Lehtinen P**, Ashorn M, Iltanen S, Jauhola R, Jauhonen P, Kolho KL, Auvinen A. Incidence trends of pediatric inflammatory bowel disease in Finland, 1987-2003, a nationwide study. *Inflamm Bowel Dis* 2011; **17**: 1778-1783 [PMID: 21744433 DOI: 10.1002/ibd.21550]

66   **Virta LJ**, Saarinen MM, Kolho KL. Inflammatory Bowel Disease Incidence is on the Continuous Rise Among All Paediatric Patients Except for the Very Young: A Nationwide Registry-based Study on 28-Year Follow-up. *J Crohns Colitis* 2017; **11**: 150-156 [PMID: 27555642 DOI: 10.1093/ecco-jcc/jjw148]

67   **Karolewska-Bochenek K**, Lazowska-Przeorek I, Albrecht P, Grzybowska K, Ryzko J, Szamotulska K, Radzikowski A, Landowski P, Krzesiek E, Ignys I, Fyderek K, Czerwionka-Szaflarska M, Jarocka-Cyrta E. Epidemiology of inflammatory bowel disease among children in Poland. A prospective, population-based, 2-year study, 2002-2004. *Digestion* 2009; **79**: 121-129 [PMID: 19321943 DOI: 10.1159/000209382]

68   **Lindberg E**, Lindquist B, Holmquist L, Hildebrand H. Inflammatory bowel disease in children and adolescents in Sweden, 1984-1995. *J Pediatr Gastroenterol Nutr* 2000; **30**: 259-264 [PMID: 10749408 DOI: 10.1097/00005176-200003000-00009]

69   **Mahdi B**, Mahdi BM. A Review Of Inflammatory Bowel Disease Unclassified --Indeterminate Colitis. *J Gastroenterol Hepatol Res* 2012; **1**: 241-246

70   **Prenzel F**, Uhlig HH. Frequency of indeterminate colitis in children and adults with IBD - a metaanalysis. *J Crohns Colitis* 2009; **3**: 277-281 [PMID: 21172287 DOI: 10.1016/j.crohns.2009.07.001]

71   **Legaki E**, Gazouli M. Influence of environmental factors in the development of inflammatory bowel diseases. *World J Gastrointest Pharmacol Ther* 2016; **7**: 112-125 [PMID: 26855817 DOI: 10.4292/wjgpt.v7.i1.112]

72   **Kamm MA**. Rapid changes in epidemiology of inflammatory bowel disease. *Lancet* 2018; **390**: 2741-2742 [PMID: 29050647 DOI: 10.1016/S0140-6736(17)32669-7]

73   **M'Koma AE**. Inflammatory bowel disease: an expanding global health problem. *Clin Med Insights Gastroenterol* 2013; **6**: 33-47 [PMID: 24833941 DOI: 10.4137/CGast.S12731]

74   **Leach ST**, Day AS, Moore D, Lemberg DA. Low rate of inflammatory bowel disease in the Australian indigenous paediatric population. *J Paediatr Child Health* 2014; **50**: 328-329 [PMID: 24698068 DOI: 10.1111/jpc.12535]

75   **Gismera CS**, Aladrén BS. Inflammatory bowel diseases: a disease (s) of modern times? Is incidence still increasing? *World J Gastroenterol* 2008; **14**: 5491-5498 [PMID: 18810764 DOI: 10.3748/WJG.14.5491]

76   **Herrinton LJ**, Liu L, Lewis JD, Griffin PM, Allison J. Incidence and prevalence of inflammatory bowel disease in a Northern California managed care organization, 1996-2002. *Am J Gastroenterol* 2008; **103**: 1998-2006 [PMID: 18796097 DOI: 10.1111/j.1572-0241.2008.01960.x]

77   **Shapiro JM**, Zoega H, Shah SA, Bright RM, Mallette M, Moniz H, Grabert SA, Bancroft B, Merrick M, Flowers NT, Samad Z, Lidofsky S, LeLeiko NS, Sands BE. Incidence of Crohn's Disease and Ulcerative Colitis in Rhode Island: Report from the Ocean State Crohn's and Colitis Area Registry. *Inflamm Bowel Dis* 2016; **22**: 1456-1461 [PMID: 26926039 DOI: 10.1097/MIB.0000000000000745]

78   **Bitton A**, Vutcovici M, Patenaude V, Sewitch M, Suissa S, Brassard P. Epidemiology of inflammatory bowel disease in Québec: recent trends. *Inflamm Bowel Dis* 2014; **20**: 1770-1776 [PMID: 25159452 DOI: 10.1097/MIB.0000000000000162]

79   **El-Matary W**, Moroz SP, Bernstein CN. Inflammatory bowel disease in children of Manitoba: 30 years' experience of a tertiary center. *J Pediatr Gastroenterol Nutr* 2014; **59**: 763-766 [PMID: 25111222 DOI: 10.1097/MPG.0000000000000525]

80   **Lowe AM**, Roy PO, B-Poulin M, Michel P, Bitton A, St-Onge L, Brassard P. Epidemiology of Crohn's disease in Québec, Canada. *Inflamm Bowel Dis* 2009; **15**: 429-435 [PMID: 18942744 DOI: 10.1002/ibd.20756]

81   **Grieci T**, Bütter A. The incidence of inflammatory bowel disease in the pediatric population of Southwestern Ontario. *J Pediatr Surg* 2009; **44**: 977-980 [PMID: 19433182 DOI: 10.1016/j.jpedsurg.2009.01.038]

82   **Griffiths AM**. Specificities of inflammatory bowel disease in childhood. *Best Pract Res Clin Gastroenterol* 2004; **18**: 509-523 [PMID: 15157824 DOI: 10.1016/j.bpg.2004.01.002]

83   **Leddin D**, Tamim H, Levy AR. Decreasing incidence of inflammatory bowel disease in eastern Canada: a population

database study. *BMC Gastroenterol* 2014; **14**: 140 [PMID: 25108544 DOI: 10.1186/1471-230X-14-140]

84   **Benchimol EI**, Manuel DG, Guttmann A, Nguyen GC, Mojaverian N, Quach P, Mack DR. Changing age demographics of inflammatory bowel disease in Ontario, Canada: a population-based cohort study of epidemiology trends. *Inflamm Bowel Dis* 2014; **20**: 1761-1769 [PMID: 25159453 DOI: 10.1097/MIB.0000000000000103]

85   **Bentsen BS**, Moum B, Ekbom A. Incidence of inflammatory bowel disease in children in southeastern Norway: a prospective population-based study 1990-94. *Scand J Gastroenterol* 2002; **37**: 540-545 [PMID: 12059055 DOI: 10.1080/00365520252903080]

86   **Benchimol EI**, Mack DR, Nguyen GC, Snapper SB, Li W, Mojaverian N, Quach P, Muise AM. Incidence, outcomes, and health services burden of very early onset inflammatory bowel disease. *Gastroenterology* 2014; **147**: 803-813.e7; quiz e14-e15 [PMID: 24951840 DOI: 10.1053/j.gastro.2014.06.023]

87   **Vicentín R**, Wagener M, Pais AB, Contreras M, Orsi M. One-year prospective registry of inflammatory bowel disease in the Argentine pediatric population. *Arch Argent Pediatr* 2017; **115**: 533-540 [PMID: 29087106 DOI: 10.5546/aap.2017.eng.533]

88   **Pebody RG**, Paunio M, Ruutu P. Measles, measles vaccination, and Crohn's disease. Crohn's disease has not increased in Finland. *BMJ* 1998; **316**: 1745-1746 [PMID: 9652932 DOI: 10.1136/bmj.316.7146.1745]

89   **Lehtinen P**, Pasanen K, Kolho KL, Auvinen A. Incidence of Pediatric Inflammatory Bowel Disease in Finland: An Environmental Study. *J Pediatr Gastroenterol Nutr* 2016; **63**: 65-70 [PMID: 26636498 DOI: 10.1097/MPG.0000000000001050]

90   **Jussila A**, Virta LJ, Kautiainen H, Rekiaro M, Nieminen U, Färkkilä MA. Increasing incidence of inflammatory bowel diseases between 2000 and 2007: a nationwide register study in Finland. *Inflamm Bowel Dis* 2012; **18**: 555-561 [PMID: 21425214 DOI: 10.1002/ibd.21695]

91   **Solberg IC**, Vatn MH, Høie O, Stray N, Sauar J, Jahnsen J, Moum B, Lygren I; IBSEN Study Group. Clinical course in Crohn's disease: results of a Norwegian population-based ten-year follow-up study. *Clin Gastroenterol Hepatol* 2007; **5**: 1430-1438 [PMID: 18054751 DOI: 10.1016/j.cgh.2007.09.002]

92   **Perminow G**, Brackmann S, Lyckander LG, Franke A, Borthne A, Rydning A, Aamodt G, Schreiber S, Vatn MH; IBSEN-II Group. A characterization in childhood inflammatory bowel disease, a new population-based inception cohort from South-Eastern Norway, 2005-07, showing increased incidence in Crohn's disease. *Scand J Gastroenterol* 2009; **44**: 446-456 [PMID: 19117240 DOI: 10.1080/00365520802647434]

93   **Størdal K**, Jahnsen J, Bentsen BS, Moum B. Pediatric inflammatory bowel disease in southeastern Norway: a five-year follow-up study. *Digestion* 2004; **70**: 226-230 [PMID: 15627770 DOI: 10.1159/000082893]

94   **Moum B**, Vatn MH, Ekbom A, Fausa O, Aadland E, Lygren I, Sauar J, Schulz T. Incidence of inflammatory bowel disease in southeastern Norway: evaluation of methods after 1 year of registration. Southeastern Norway IBD Study Group of Gastroenterologists. *Digestion* 1995; **56**: 377-381 [PMID: 8549880 DOI: 10.1159/000201262]

95   **Moum B**, Vatn MH, Ekbom A, Aadland E, Fausa O, Lygren I, Sauar J, Schulz T, Stray N. Incidence of ulcerative colitis and indeterminate colitis in four counties of southeastern Norway, 1990-93. A prospective population-based study. The Inflammatory Bowel South-Eastern Norway (IBSEN) Study Group of Gastroenterologists. *Scand J Gastroenterol* 1996; **31**: 362-366 [PMID: 8726304 DOI: 10.3109/00365529609006411]

96   **Moum B**, Vatn MH, Ekbom A, Aadland E, Fausa O, Lygren I, Stray N, Sauar J, Schulz T. Incidence of Crohn's disease in four counties in southeastern Norway, 1990-93. A prospective population-based study. The Inflammatory Bowel South-Eastern Norway (IBSEN) Study Group of Gastroenterologists. *Scand J Gastroenterol* 1996; **31**: 355-361 [PMID: 8726303 DOI: 10.3109/00365529609006410]

97   **Olafsdottir EJ**, Fluge G, Haug K. Chronic inflammatory bowel disease in children in western Norway. *J Pediatr Gastroenterol Nutr* 1989; **8**: 454-458 [PMID: 2723937 DOI: 10.1097/00005176-19890 5000-00006]

98   **Lindberg E**, Jörnerot G. The incidence of Crohn's disease is not decreasing in Sweden. *Scand J Gastroenterol* 1991; **26**: 495-500 [PMID: 1871542 DOI: 10.3109/00365529108998572]

99   **Askling J**, Grahnquist L, Ekbom A, Finkel Y. Incidence of paediatric Crohn's disease in Stockholm, Sweden. *Lancet* 1999; **354**: 1179 [PMID: 10513717 DOI: 10.1016/S0140-6736(99)02625-2]

100   **Sjöberg D**, Holmström T, Larsson M, Nielsen AL, Holmquist L, Ekbom A, Rönnblom A. Incidence and natural history of ulcerative colitis in the Uppsala Region of Sweden 2005-2009 - results from the IBD cohort of the Uppsala Region (ICURE). *J Crohns Colitis* 2013; **7**: e351-e357 [PMID: 23491313 DOI: 10.1016/j.crohns.2013.02.006]

101   **Sjöberg D**, Holmström T, Larsson M, Nielsen AL, Holmquist L, Ekbom A, Rönnblom A. Incidence and clinical course of Crohn's disease during the first year - results from the IBD Cohort of the Uppsala Region (ICURE) of Sweden 2005-2009. *J Crohns Colitis* 2014; **8**: 215-222 [PMID: 24035547 DOI: 10.1016/j.crohns.2013.08.009]

102   **Vind I**, Riis L, Jess T, Knudsen E, Pedersen N, Elkjaer M, Bak Andersen I, Wewer V, Nørregaard P, Moesgaard F, Bendtsen F, Munkholm P; DCCD study group. Increasing incidences of inflammatory bowel disease and decreasing surgery rates in Copenhagen City and County, 2003-2005: a population-based study from the Danish Crohn colitis database. *Am J Gastroenterol* 2006; **101**: 1274-1282 [PMID: 16771949 DOI: 10.1111/j.1572-0241.2006.00552.x]

103   **Lophaven SN**, Lynge E, Burisch J. The incidence of inflammatory bowel disease in Denmark 1980-2013: a nationwide cohort study. *Aliment Pharmacol Ther* 2017; **45**: 961-972 [PMID: 28229470 DOI: 10.1111/apt.13971]

104   **Jacobsen BA**, Fallingborg J, Rasmussen HH, Nielsen KR, Drewes AM, Puho E, Nielsen GL, Sørensen HT. Increase in incidence and prevalence of inflammatory bowel disease in northern Denmark: a population-based study, 1978-2002. *Eur J Gastroenterol Hepatol* 2006; **18**: 601-606 [PMID: 16702848 DOI: 10.1097/00042737-200 606000-00005]

105   **Langholz E**, Munkholm P, Krasilnikoff PA, Binder V. Inflammatory bowel diseases with onset in childhood. Clinical features, morbidity, and mortality in a regional cohort. *Scand J Gastroenterol* 1997; **32**: 139-147 [PMID: 9051874 DOI: 10.3109/00365529709000184]

106   **Langholz E**, Munkholm P, Nielsen OH, Kreiner S, Binder V. Incidence and prevalence of ulcerative colitis in Copenhagen county from 1962 to 1987. *Scand J Gastroenterol* 1991; **26**: 1247-1256 [PMID: 1763295 DOI: 10.3109/00365529108998621]

107   **Fonager K**, Sørensen HT, Olsen J. Change in incidence of Crohn's disease and ulcerative colitis in Denmark. A study based on the National Registry of Patients, 1981-1992. *Int J Epidemiol* 1997; **26**: 1003-1008 [PMID: 9363521 DOI: 10.1093/ije/26.5.1003]

108   **Urne FU**, Paerregaard A. [Chronic inflammatory bowel disease in children. An epidemiological study from eastern Denmark 1998-2000]. *Ugeskr Laeger* 2002; **164**: 5810-5814 [PMID: 12523226]

109   **Larsen L**, Jensen MD, Larsen MD, Nielsen RG, Thorsgaard N, Vind I, Wildt S, Kjeldsen J. The Danish National Registry for Biological Therapy in Inflammatory Bowel Disease. *Clin Epidemiol* 2016; **8**: 607-612 [PMID: 27822107 DOI: 10.2147/CLEP.S99478]

110   **Jakobsen C**, Paerregaard A, Munkholm P, Faerk J, Lange A, Andersen J, Jakobsen M, Kramer I, Czernia-Mazurkiewicz J, Wewer V. Pediatric inflammatory bowel disease: increasing incidence, decreasing surgery rate, and compromised nutritional status: A prospective population-based cohort study 2007-2009. *Inflamm Bowel Dis* 2011; **17**: 2541-2550 [PMID: 21381152 DOI: 10.1002/ibd.21654]

111   **Nørgård BM**, Nielsen J, Fonager K, Kjeldsen J, Jacobsen BA, Qvist N. The incidence of ulcerative colitis (1995-2011) and Crohn's disease (1995-2012) - based on nationwide Danish registry data. *J Crohns Colitis* 2014; **8**: 1274-1280 [PMID: 24675473 DOI: 10.1016/j.crohns.2014.03.006]

112   **Björnsson S**, Jóhannsson JH. Inflammatory bowel disease in Iceland, 1990-1994: a prospective, nationwide, epidemiological study. *Eur J Gastroenterol Hepatol* 2000; **12**: 31-38 [PMID: 10656207 DOI: 10.1097/00042737-200012010-00007]

113   **Armitage E**, Drummond H, Ghosh S, Ferguson A. Incidence of juvenile-onset Crohn's disease in Scotland. *Lancet* 1999; **353**: 1496-1497 [PMID: 10232323 DOI: 10.1016/S0140-6736(99)00333-5]

114   **Armitage EL**, Aldhous MC, Anderson N, Drummond HE, Riemersma RA, Ghosh S, Satsangi J. Incidence of juvenile-onset Crohn's disease in Scotland: association with northern latitude and affluence. *Gastroenterology* 2004; **127**: 1051-1057 [PMID: 15480983 DOI: 10.1053/j.gastro.2004.06.024]

115   **Watson AJ**, Johnston AT, Barker PM, Youngson GG, Bisset WM, Mahomed AA. The presentation and management of juvenile-onset chronic inflammatory bowel disease in Northeastern Scotland. *J Pediatr Surg* 2002; **37**: 83-86 [PMID: 11781993 DOI: 10.1053/jpsu.2002.29434]

116   **Ashton JJ**, Wiskin AE, Ennis S, Batra A, Afzal NA, Beattie RM. Rising incidence of paediatric inflammatory bowel disease (PIBD) in Wessex, Southern England. *Arch Dis Child* 2014; **99**: 659-664 [PMID: 24728445 DOI: 10.1136/archdischild-2013-305419]

117   **Ashton JJ**, Cullen M, Afzal NA, Coelho T, Batra A, Beattie RM. Is the incidence of paediatric inflammatory bowel disease still increasing? *Arch Dis Child* 2018; Epub ahead of print [PMID: 29519945 DOI: 10.1136/archdischild-2018-315038]

118   **Hassan K**, Cowan FJ, Jenkins HR. The incidence of childhood inflammatory bowel disease in Wales. *Eur J Pediatr* 2000; **159**: 261-263 [PMID: 10789930 DOI: 10.1007/s004310050066]

119   **Gunesh S**, Thomas GA, Williams GT, Roberts A, Hawthorne AB. The incidence of Crohn's disease in Cardiff over the last 75 years: an update for 1996-2005. *Aliment Pharmacol Ther* 2008; **27**: 211-219 [PMID: 18005244 DOI: 10.1111/j.1365-2036.2007.03576.x]

120   **Hope B**, Shahdadpuri R, Dunne C, Broderick AM, Grant T, Hamzawi M, O'Driscoll K, Quinn S, Hussey S, Bourke B. Rapid rise in incidence of Irish paediatric inflammatory bowel disease. *Arch Dis Child* 2012; **97**: 590-594 [PMID: 22550323 DOI: 10.1136/archdischild-2011-300651]

121   **Coughlan A**, Wylde R, Lafferty L, Quinn S, Broderick A, Bourke B, Hussey S; DOCHAS Study. A rising incidence and poorer male outcomes characterise early onset paediatric inflammatory bowel disease. *Aliment Pharmacol Ther* 2017; **45**: 1534-1541 [PMID: 28449214 DOI: 10.1111/apt.14070]

122   **Petritsch W**, Fuchs S, Berghold A, Bachmaier G, Högenauer C, Hauer AC, Weiglhofer U, Wenzl HH. Incidence of inflammatory bowel disease in the province of Styria, Austria, from 1997 to 2007: a population-based study. *J Crohns Colitis* 2013; **7**: 58-69 [PMID: 22542057 DOI: 10.1016/j.crohns.2012.03.012]

123   **Ott C**, Obermeier F, Thieler S, Kemptner D, Bauer A, Schölmerich J, Rogler G, Timmer A. The incidence of inflammatory bowel disease in a rural region of Southern Germany: a prospective population-based study. *Eur J Gastroenterol Hepatol* 2008; **20**: 917-923 [PMID: 18794607 DOI: 10.1097/MEG.0b013e3282f97b33]

124   **van der Zaag-Loonen HJ**, Casparie M, Taminiau JA, Escher JC, Pereira RR, Derkx HH. The incidence of pediatric inflammatory bowel disease in the Netherlands: 1999-2001. *J Pediatr Gastroenterol Nutr* 2004; **38**: 302-307 [PMID: 15076631 DOI: 10.1097/00005176-200403000-00014]

125   **Gower-Rousseau C**, Vasseur F, Fumery M, Savoye G, Salleron J, Dauchet L, Turck D, Cortot A, Peyrin-Biroulet L, Colombel JF. Epidemiology of inflammatory bowel diseases: new insights from a French population-based registry (EPIMAD). *Dig Liver Dis* 2013; **45**: 89-94 [PMID: 23107487 DOI: 10.1016/j.dld.2012.09.005]

126   **Bequet E**, Sarter H, Fumery M, Vasseur F, Armengol-Debeir L, Pariente B, Ley D, Spyckerelle C, Coevoet H, Laberenne JE, Peyrin-Biroulet L, Savoye G, Turck D, Gower-Rousseau C; EPIMAD Group. Incidence and Phenotype at Diagnosis of Very-early-onset Compared with Later-onset Paediatric Inflammatory Bowel Disease: A Population-based Study [1988-2011]. *J Crohns Colitis* 2017; **11**: 519-526 [PMID: 28453757 DOI: 10.1093/ecco-jcc/jjw194]

127   **Vernier-Massouille G**, Balde M, Salleron J, Turck D, Dupas JL, Mouterde O, Merle V, Salomez JL, Branche J, Marti R, Lerebours E, Cortot A, Gower-Rousseau C, Colombel JF. Natural history of pediatric Crohn's disease: a population-based cohort study. *Gastroenterology* 2008; **135**: 1106-1113 [PMID: 18692056 DOI: 10.1053/j.gastro.2008.06.079]

128   **Abakar-Mahamat A**, Filippi J, Pradier C, Dozol A, Hébuterne X. Incidence of inflammatory bowel disease in Corsica from 2002 to 2003. *Gastroenterol Clin Biol* 2007; **31**: 1098-1103 [PMID: 18176365 DOI: 10.1016/S0399-8320(07)78343-4]

129   **Tourtelier Y,** Dabadie A, Tron I, Alexandre JL, Robaskiewicz M, Cruchant E, Seyrig JA, Heresbach D, Bretagne JF. Incidence of inflammatory bowel disease in children in Brittany (1994-1997). *Arch Pediatr* 2000; **7**: 377-384 [DOI: 10.1016/s0929-693x(00)88832-6]

130   **Gottrand F**, Colombel JF, Moreno L, Salomez JL, Farriaux JP, Cortot A. [Incidence of inflammatory bowel diseases in children in the Nord-Pas-de-Calais region]. *Arch Fr Pediatr* 1991; **48**: 25-28 [PMID: 2018416]

131   **Ghione S**, Sarter H, Fumery M, Armengol-Debeir L, Savoye G, Ley D, Spyckerelle C, Pariente B, Peyrin-Biroulet L, Turck D, Gower-Rousseau C; Epimad Group. Dramatic Increase in Incidence of Ulcerative Colitis and Crohn's Disease (1988-2011): A Population-Based Study of French Adolescents. *Am J Gastroenterol* 2018; **113**: 265-272 [PMID: 28809388 DOI: 10.1038/ajg.2017.228]

132   **Pittet V**, Juillerat P, Mottet C, Felley C, Ballabeni P, Burnand B, Michetti P, Vader JP; Swiss IBD Cohort Study Group. Cohort profile: the Swiss Inflammatory Bowel Disease Cohort Study (SIBDCS). *Int J Epidemiol* 2009; **38**: 922-931 [PMID: 18782896 DOI: 10.1093/ije/dyn180]

133   **De Greef E**, Mahachie John JM, Hoffman I, Smets F, Van Biervliet S, Scaillon M, Hauser B, Paquot I, Alliet P, Arts W, Dewit O, Peeters H, Baert F, D'Haens G, Rahier JF, Etienne I, Bauraind O, Van Gossum A, Vermeire S, Fontaine F, Muls V, Louis E, Van de Mierop F, Coche JC, Van Steen K, Veereman G; IBD working group of the Belgian Society of Pediatric Gastroenterology, Hepatology and Nutrition (BeSPGHAN); Belgian IBD Research and Development. Profile of pediatric Crohn's disease in Belgium. *J Crohns Colitis* 2013; **7**: e588-e598 [PMID: 23664896 DOI: 10.1016/j.crohns.2013.04.016]

134   **Ranzi T**, Bodini P, Zambelli A, Politi P, Lupinacci G, Campanini MC, Dal Lago AL, Lisciandrano D, Bianchi PA. Epidemiological aspects of inflammatory bowel disease in a north Italian population: a 4-year prospective study. *Eur J Gastroenterol Hepatol* 1996; **8**: 657-661 [PMID: 8853254]

135   **Di Domenicantonio R**, Cappai G, Arcà M, Agabiti N, Kohn A, Vernia P, Biancone L, Armuzzi A, Papi C, Davoli M. Occurrence of inflammatory bowel disease in central Italy: a study based on health information systems. *Dig Liver Dis* 2014; **46**: 777-782 [PMID: 24890621 DOI: 10.1016/j.dld.2014.04.014]

136   **Trallori G**, Palli D, Saieva C, Bardazzi G, Bonanomi AG, d'Albasio G, Galli M, Vannozzi G, Milla M, Tarantino O, Renai F, Messori A, Amorosi A, Pacini F, Morettini A. A population-based study of inflammatory bowel disease in Florence over 15 years (1978-92). *Scand J Gastroenterol* 1996; **31**: 892-899 [PMID: 8888437 DOI: 10.3109/00365529609051998]

137   **Arin Letamendia A**, Borda Celaya F, Burusco Paternain MJ, Prieto Martínez C, Martínez Echeverría A, Elizalde Apestegui I, Laiglesia Izquierdo M, Macias Mendizábal E, Tamburri Moso P, Sánchez Valverde F. [High incidence rates of inflammatory bowel disease in Navarra (Spain). Results of a prospective, population-based study]. *Gastroenterol Hepatol* 2008; **31**: 111-116 [PMID: 18341841 DOI: 10.1157/13116497]

138   **Cachia E**, Calleja N, Aakeroy R, Degaetano J, Vassallo M. Incidence of inflammatory bowel disease in Malta between 1993 and 2005: a retrospective study. *Inflamm Bowel Dis* 2008; **14**: 550-553 [PMID: 18183599 DOI: 10.1002/ibd.20321]

139   **Pozler O**, Maly J, Bonova O, Dedek P, Frühauf P, Havlickova A,

Janatova T, Jimramovsky F, Klimova L, Klusacek D, Kocourkova D, Kolek A, Kotalova R, Marx D, Nevoral J, Petro R, Petru O, Plasilova I, Seidl Z, Sekyrova I, Semendak N, Schreierova I, Stanek J, Sykora J, Sulakova A, Toukalkova L, Travnickova R, Volf V, Zahradnicek L, Zenisková I. Incidence of Crohn disease in the Czech Republic in the years 1990 to 2001 and assessment of pediatric population with inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 2006; **42**: 186-189 [PMID: 16456413 DOI: 10.1097/01.mpg.0000189328.47150.bc]

140   **Kolek A**, Janout V, Tichý M, Grepl M. The incidence of inflammatory bowel disease is increasing among children 15 years old and younger in the Czech Republic. *J Pediatr Gastroenterol Nutr* 2004; **38**: 362-363 [PMID: 15076645 DOI: 10.1097/00005176-200403000-00028]

141   **Müller KE**, Lakatos PL, Arató A, Kovács JB, Várkonyi Á, Szűcs D, Szakos E, Sólyom E, Kovács M, Polgár M, Nemes É, Guthy I, Tokodi I, Tóth G, Horváth Á, Tárnok A, Csoszánszki N, Balogh M, Vass N, Bódi P, Dezsőfi A, Gárdos L, Micskey E, Papp M, Cseh Á, Szabó D, Vörös P, Veres G; Hungarian IBD Registry Group (HUPIR). Incidence, Paris classification, and follow-up in a nationwide incident cohort of pediatric patients with inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 2013; **57**: 576-582 [PMID: 23820399 DOI: 10.1097/MPG.0b013e31829f7d8c]

142   **Lakatos L**, Kiss LS, David G, Pandur T, Erdelyi Z, Mester G, Balogh M, Szipocs I, Molnar C, Komaromi E, Lakatos PL. Incidence, disease phenotype at diagnosis, and early disease course in inflammatory bowel diseases in Western Hungary, 2002-2006. *Inflamm Bowel Dis* 2011; **17**: 2558-2565 [PMID: 22072315 DOI: 10.1002/ibd.21607]

143   **Orel R**, Kamhi T, Vidmar G, Mamula P. Epidemiology of pediatric chronic inflammatory bowel disease in central and western Slovenia, 1994-2005. *J Pediatr Gastroenterol Nutr* 2009; **48**: 579-586 [PMID: 19367184 DOI: 10.1097/MPG.0b013e318164d903]

144   **Vucelić B**, Korać B, Sentić M, Milicić D, Hadžić N, Juresa V, Bozikov J, Rotkvić I, Buljevac M, Kovacević I. Ulcerative colitis in Zagreb, Yugoslavia: incidence and prevalence 1980-1989. *Int J Epidemiol* 1991; **20**: 1043-1047 [PMID: 1800402 DOI: 10.1093/ije/20.4.1043]

145   **Vucelić B**, Korać B, Sentić M, Milicić D, Hadžić N, Juresa V, Bozikov J, Rotkvić I, Buljevac M, Kovacević I. Epidemiology of Crohn's disease in Zagreb, Yugoslavia: a ten-year prospective study. *Int J Epidemiol* 1991; **20**: 216-220 [PMID: 2066223 DOI: 10.1093/ije/20.1.216]

146   **Ahmaida A**, Al-Shaikhi S. Childhood Inflammatory Bowel Disease in Libya: Epidemiological and Clinical features. *Libyan J Med* 2009; **4**: 70-74 [PMID: 21483512 DOI: 10.4176/081210]

147   **Odes HS**, Locker C, Neumann L, Zirkin HJ, Weizman Z, Sperber AD, Fraser GM, Krugliak P, Gaspar N, Eidelman L. Epidemiology of Crohn's disease in southern Israel. *Am J Gastroenterol* 1994; **89**: 1859-1862 [PMID: 7942683]

148   **Grossman A**, Fireman Z, Lilos P, Novis B, Rozen P, Gilat T. Epidemiology of ulcerative colitis in the Jewish population of central Israel 1970-1980. *Hepatogastroenterology* 1989; **36**: 193-197 [PMID: 2807136]

149   **El Mouzan MI**, Saadah O, Al-Saleem K, Al Edreesi M, Hasosah M, Alanazi A, Al Mofarreh M, Asery A, Al Qourain A, Nouli K, Al Hussaini A, Telmesani A, AlReheili K, Alghamdi S, Alrobiaa N, Alzaben A, Mehmadi A, Al Hebbi H, Al Sarkhy A, Al Mehaidib A, Al Saleem K, Assiri A, Wali S. Incidence of pediatric inflammatory bowel disease in Saudi Arabia: a multicenter national study. *Inflamm Bowel Dis* 2014; **20**: 1085-1090 [PMID: 24788219 DOI: 10.1097/MIB.0000000000000048]

150   **El Mouzan MI**, Abdullah AM, Al Habbal MT. Epidemiology of juvenile-onset inflammatory bowel disease in central Saudi Arabia. *J Trop Pediatr* 2006; **52**: 69-71 [PMID: 15947013 DOI: 10.1093/tropej/fmi039]

151   **Al-Qabandi WA**, Buhamrah EK, Hamadi KA, Al-Osaimi SA, Al-Ruwayeh AA, Madda J. Inflammatory bowel disease in children, an evolving problem in Kuwait. *Saudi J Gastroenterol* 2011; **17**: 323-327 [PMID: 21912059 DOI: 10.4103/1319-3767.84487]

152   **Zayyani NR**, Malaty HM, Graham DY. Increasing Incidence of Crohn's Disease with Familial Clustering in the Kingdom of Bahrain: A 25-Year Population-based Study. *Inflamm Bowel Dis* 2017; **23**: 304-309 [PMID: 28092308 DOI: 10.1097/MIB.0000000000001016]

153   **Wang XQ**, Zhang Y, Xu CD, Jiang LR, Huang Y, Du HM, Wang XJ. Inflammatory bowel disease in Chinese children: a multicenter analysis over a decade from Shanghai. *Inflamm Bowel Dis* 2013; **19**: 423-428 [PMID: 23340680 DOI: 10.1097/MIB.0b013e318286f9f2]

154   **Tsai CH**, Chen HL, Ni YH, Hsu HY, Jeng YM, Chang CJ, Chang MH. Characteristics and trends in incidence of inflammatory bowel disease in Taiwanese children. *J Formos Med Assoc* 2004; **103**: 685-691 [PMID: 15361941]

155   **Chu HP**, Logarajah V, Tan N, Phua KB. Paediatric inflammatory bowel disease in a multiracial Asian country. *Singapore Med J* 2013; **54**: 201-205 [PMID: 23624446 DOI: 10.11622/smedj.2013073]

156   **Yang SK**, Hong WS, Min YI, Kim HY, Yoo JY, Rhee PL, Rhee JC, Chang DK, Song IS, Jung SA, Park EB, Yoo HM, Lee DK, Kim YK. Incidence and prevalence of ulcerative colitis in the Songpa-Kangdong District, Seoul, Korea, 1986-1997. *J Gastroenterol Hepatol* 2000; **15**: 1037-1042 [PMID: 11059934 DOI: 10.1046/j.1440-1746.2000.02252.x]

157   **Jung YS**, Han M, Kim WH, Park S, Cheon JH. Incidence and Clinical Outcomes of Inflammatory Bowel Disease in South Korea, 2011-2014: A Nationwide Population-Based Study. *Dig Dis Sci* 2017; **62**: 2102-2112 [PMID: 28593437 DOI: 10.1007/s10620-017-4640-9]

158   **Phavichitr N**, Cameron DJ, Catto-Smith AG. Increasing incidence of Crohn's disease in Victorian children. *J Gastroenterol Hepatol* 2003; **18**: 329-332 [PMID: 12603535 DOI: 10.1046/j.1440-1746.2003.02975.x]

159   **Schildkraut V**, Alex G, Cameron DJ, Hardikar W, Lipschitz B, Oliver MR, Simpson DM, Catto-Smith AG. Sixty-year study of incidence of childhood ulcerative colitis finds eleven-fold increase beginning in 1990s. *Inflamm Bowel Dis* 2013; **19**: 1-6 [PMID: 22532319 DOI: 10.1002/ibd.22997]

160   **Lopez RN**, Evans HM, Appleton L, Bishop J, Chin S, Mouat S, Gearry RB, Day AS. Prospective Incidence of Paediatric Inflammatory Bowel Disease in New Zealand in 2015: Results From the Paediatric Inflammatory Bowel Disease in New Zealand (PINZ) Study. *J Pediatr Gastroenterol Nutr* 2018; **66**: e122-e126 [PMID: 29077643 DOI: 10.1097/MPG.0000000000001806]

161   **Lopez RN**, Appleton L, Gearry RB, Day AS. Rising Incidence of Paediatric Inflammatory Bowel Disease in Canterbury, New Zealand, 1996-2015. *J Pediatr Gastroenterol Nutr* 2018; **66**: e45-e50 [PMID: 28727653 DOI: 10.1097/MPG.0000000000001688]

162   **Levine A**, Koletzko S, Turner D, Escher JC, Cucchiara S, de Ridder L, Kolho KL, Veres G, Russell RK, Paerregaard A, Buderus S, Greer ML, Dias JA, Veereman-Wauters G, Lionetti P, Sladek M, Martin de Carpi J, Staiano A, Ruemmele FM, Wilson DC; European Society of Pediatric Gastroenterology, Hepatology, and Nutrition. ESPGHAN revised porto criteria for the diagnosis of inflammatory bowel disease in children and adolescents. *J Pediatr Gastroenterol Nutr* 2014; **58**: 795-806 [PMID: 24231644 DOI: 10.1097/MPG.0000000000000239]

163   **Haug K**, Schrumpf E, Barstad S, Fluge G, Halvorsen JF. Epidemiology of ulcerative colitis in western Norway. *Scand J Gastroenterol* 1988; **23**: 517-522 [PMID: 3399823 DOI: 10.3109/00365528809093904]

164   **Hildebrand H**, Brydolf M, Holmquist L, Krantz I, Kristiansson B. Incidence and prevalence of inflammatory bowel disease in children in south-western Sweden. *Acta Paediatr* 1994; **83**: 640-645 [PMID: 7919763 DOI: 10.1111/j.1651-2227.1994.tb13098.x]

165   **Tysk C**, Järnerot G. Ulcerative proctocolitis in Orebro, Sweden. A retrospective epidemiologic study, 1963-1987. *Scand J Gastroenterol* 1992; **27**: 945-950 [PMID: 1455192 DOI: 10.3109/00365529209000168]

166   **Hildebrand H**, Fredrikzon B, Holmquist L, Kristiansson B, Lindquist B. Chronic inflammatory bowel disease in children and adolescents in Sweden. *J Pediatr Gastroenterol Nutr* 1991; **13**:

293-297 [PMID: 1791507 DOI: 10.1097/00005176-199110000-000 10]

167 **Gollop JH**, Phillips SF, Melton LJ 3rd, Zinsmeister AR. Epidemiologic aspects of Crohn's disease: a population based study in Olmsted County, Minnesota, 1943-1982. *Gut* 1988; **29**: 49-56 [PMID: 3343012 DOI: 10.1136/gut.29.1.49]

168 **Loftus EV Jr**, Silverstein MD, Sandborn WJ, Tremaine WJ, Harmsen WS, Zinsmeister AR. Ulcerative colitis in Olmsted County, Minnesota, 1940-1993: incidence, prevalence, and survival. *Gut* 2000; **46**: 336-343 [PMID: 10673294 DOI: 10.1136/gut.46.3.336]

169 **Kuo CJ**, Yu KH, See LC, Chiu CT, Su MY, Hsu CM, Kuo CF, Chiou MJ, Liu JR, Wang HW. The Trend of Inflammatory Bowel Diseases in Taiwan: A Population-Based Study. *Dig Dis Sci* 2015; **60**: 2454-2462 [PMID: 25837597 DOI: 10.1007/s10620-015-3630-z]

170 **González FN,** García BC, Polo RE, Hernández CM. Inflammatory bowel disease in pediatric patients in Asturias, Spain (1993-2000): epidemiology and clinical features. *Acta Pediatr Esp* 2004; **62**: 466-472

171 **Lopez Miguel C**, Sicilia B, Sierra E, Lopez Zaborras J, Arribas F, Gomollon F. [Incidence of inflammatory bowel disease in Aragon: outcome of a prospective population-based study]. *Gastroenterol Hepatol* 1999; **22**: 323-328 [PMID: 10535203]

META-ANALYSIS

Systematic review and meta-analysis on the association of tuberculosis in Crohn's disease patients treated with tumor necrosis factor-α inhibitors (Anti-TNFα)

Brent L Cao, Ahmad Qasem, Robert C Sharp, Latifa S Abdelli, Saleh A Naser

Brent L Cao, Ahmad Qasem, Robert C Sharp, Latifa S Abdelli, Saleh A Naser, Division of Molecular Microbiology, Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, FL 32816, United States

ORCID number: Brent L Cao (0000-0002-6148-6409); Ahmad Qasem (0000-0003-3740-6963); Robert C Sharp (0000-0002-5167-8941); Latifa S Abdelli (0000-0001-6216-3241); Saleh A Naser (0000-0001-9222-4527).

Author contributions: Cao BL and Qasem A are Co-first authors; Cao BL designed the study, acquired the data, analyzed and interpreted the data, and participating in writing the manuscript; Qasem A, Sharp RC, and Abdelli LS interpreted the data and participated in revisions of the manuscript; Naser SA is the group leader who supervised all aspects of the data collection/analyses and manuscript writing.

Conflict-of-interest statement: The authors declare that they have no competing interests.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Correspondence to: Saleh A Naser, PhD, Professor, Associate Director, Burnett School of Biomedical Sciences, Division of Molecular Microbiology, College of Medicine, University of Central Florida, 4110 Libra Drive, Orlando, FL 32816, United States. saleh.naser@ucf.edu
Telephone: +1-407-8230955
Fax: +1-407-8230955

Abstract

AIM
To perform a meta-analysis on the risk of developing *Mycobacterium tuberculosis* (TB) infection in Crohn's disease (CD) patients treated with tumor necrosis factor-alpha (TNFα) inhibitors.

METHODS
A meta-analysis of randomized, double-blind, placebo-controlled trials of TNFα inhibitors for treatment of CD in adults was conducted. Arcsine transformation of TB incidence was performed to estimate risk difference. A novel epidemiologically-based correction (EBC) enabling inclusions of studies reporting no TB infection cases in placebo and treatment groups was developed to estimate relative odds.

RESULTS
Twenty-three clinical trial studies were identified, including 5669 patients. Six TB infection cases were reported across 5 studies, all from patients receiving TNFα inhibitors. Eighteen studies reported no TB infection cases in placebo and TNFα inhibitor treatment arms. TB infection risk was significantly increased among patients receiving TNFα inhibitors, with a risk difference of 0.028 (95%CI: 0.0011-0.055). The odds ratio was 4.85 (95%CI: 1.02-22.99) with EBC and 5.85 (95%CI: 1.13-30.38) without EBC.

CONCLUSION

The risk of TB infection is higher among CD patients receiving TNFα inhibitors. Understanding the immunopathogenesis of CD is crucial, since using TNFα inhibitors in these patients could favor mycobacterial infections, particularly *Mycobacterium avium* subspecies *paratuberculosis*, which ultimately could worsen their clinical condition.

Key words: Tuberculosis; Tumor necrosis factor-alpha inhibitors; Crohn's Disease; Meta-analysis; Systematic review

Core tip: The increased risk of *Mycobacterium tuberculosis* (TB) and other *Mycobacterium* species when on tumor necrosis factor-alpha (TNFα) inhibitor treatments has been a problem in patients with autoimmune disorders, such as Crohn's disease (CD). This meta-analysis examines in detail the clinical trials that involve CD patients on TNFα inhibitors and their risk of developing TB infections. Our data concludes that, out of twenty-three studies examined, TNFα inhibitors are indeed associated with an increased risk of TB infection in CD patients. Knowledge of this data could help re-analyze what medications autoimmune patients should be prescribed to and evaluate possible linkages to *Mycobacterium avium* subspecies *paratuberculosis* infection. Thus, this information should be used to further inform clinical decision making and research.

INTRODUCTION

The prevalence of Crohn's disease (CD) is growing globally, especially in places adapting western lifestyles[1]. The most accepted hypothesis for CD pathogenesis is that a dysregulated immune response to opportunistic intestinal pathogens leads to persistent inflammation[2]. Studies have shown that the levels of circulating pro-inflammatory cytokines are highly elevated in CD patients, including tumor necrosis factor-alpha (TNFα)[3]. Therefore, there is an increase in the development and use of TNFα inhibitors, which are monoclonal antibodies designed to antagonize this specific cytokine in order to reduce the symptoms of CD[4]. Currently, there are three European Medicines Agency and United States Food and Drug Association approved TNFα inhibitors indicated for CD treatment:

Adalimumab, certolizumab pegol, and infliximab.

Although these medications have shown efficacy in alleviating CD symptoms in some patients, 10%-30% of CD patients had no initial response to TNFα inhibitors, and about 50% of the initial responders have lost their response over time[5]. TNFα inhibitor treatment for other inflammatory autoimmune disorders, such as rheumatoid arthritis (RA), show similar effects, where roughly 30%-40% of RA patients have a poor response to the medications[6,7]. Moreover, about 40% of CD patients are at high risk of disease relapse after discontinuing TNFα inhibitor treatment[8].

Additionally, inhibiting TNFα increased risk of infections as an adverse effect, especially opportunistic infections[9]. However, it is uncertain whether there is sufficient evidence indicating an increase in risk of developing *Mycobacterium tuberculosis* (TB) infection from using TNFα inhibitors among CD patients. A 2016 meta-analysis reported a significant increase in opportunistic infections overall and a moderate increase in other types of infections; however, that study did not find a significant increase in the risk of TB infection development among CD patients who received TNFα inhibitors[10].

Our primary focus on TB infection is pertinent, since there is an irrefutable growing evidence relating CD to *Mycobacterium avium* subspecies *paratuberculosis* (MAP), an intracellular TB-like bacterium[11]. More importantly, TNFα is necessary for formation and maintenance of granuloma in order to limit mycobacterial infections like MAP[12]. Thus, targeting TNFα can not only disrupt the body's ability to contain and respond to TB but also to MAP, which could further increase the patient's susceptibility to MAP or worsen their disease condition[2]. Therefore, the effect of TNFα inhibitors on CD patients-associated with MAP infection requires further investigation.

Other meta-analyses of TB infection risk potentially associated with anti-TNFα therapy excluded trials reporting zero-event data from both anti-TNFα treatment and placebo groups ("double-zero studies")[13,14]. The number of such "double-zero studies" was high as the incidence of TB infection was very low. Additionally, data from trials reporting zero-event data from one group ("single-zero studies") were subject to modification ("continuity correction") that lacks biological basis. These analytical approaches cast uncertainty about whether there is sufficient evidence indicating an increase in risk of developing TB infection from using TNFα inhibitors. Numerous double-blind, randomized, placebo-controlled trials (RCTs) of TNFα inhibitor use in CD patients provide data to estimate the risk of TB development in CD patients receiving those medications. This will open more insights into selecting the most appropriate treatment plan and antibiotics for CD patients[15]. Thus, we conducted a meta-analysis of randomized controlled trials to quantitatively analyze the altered risk for developing TB in CD patients treated with TNFα inhibitors.

MATERIALS AND METHODS

This systematic review and meta-analysis was registered in the prospective register of systematic reviews (PROSPERO) international database on February 8th, 2018 (ID: CRD42018087548)[16]. The work followed the preferred reporting items for the systematic reviews and meta-analyses (PRISMA) checklist[17].

Data source and search strategy

A search in the PubMed database up to January 21, 2018 was conducted. The following search terms were used: Tuberculosis, biologic(s), adalimumab, certolizumab, infliximab, anti-TNFα, TNFα inhibitors, or TNFα in conjunction with Crohn's disease. The search results were further restricted to double-masked, randomized, placebo-controlled trials. ClinicalTrials.gov supplemented the searches, in the event of clinical trials that did not yet have published data in PubMed. Irrelevant studies were screened out after title and abstract review. Full text and abstracts of studies that made it past the initial screening were evaluated more closely.

Selection

Only studies and sources in English were considered. Studies were included if they were randomized, placebo-controlled, double-masked trials with appropriate exposure in adult populations. Exposure was defined as the patient receiving treatment of TNFα inhibitors (adalimumab, certolizumab pegol, and infliximab), which were approved for the treatment of CD in adult patients (18 years or older) by the EMA and the FDA. Non-approved drugs and biosimilars of TNFα inhibitors were excluded from the study. All doses of drugs were included. Observational studies and duplicates were screened out. Single studies that had both an induction and maintenance phase but reported distinct patient groups were analyzed as two separate trials.

Data extraction

Data was extracted onto a Microsoft Excel spreadsheet. First author, year of publication, study duration, number of participants in treatment and control groups, patient characteristics, treatment parameters (*i.e.*, TNFα inhibitor and placebo), events in treatment and control groups, and screening method were obtained from each study. Studies found from ClinicalTrials.gov were also analyzed for the aforementioned criteria. Cases of TB infection were the primary outcome assessed in this analysis. TB infection was defined as diagnosis of active TB by the clinician or other medical professional.

Risk difference

Arcsine differences (ASD) were used as the measure of risk differences. For a trial with N_T subjects in the anti-TNFα treatment group, N_C subjects in the control group, and a and b being the number of reported TB cases, respectively, the ASD can be calculated with Equation 1 below:

$$\widehat{ASD} = \arcsin\sqrt{\frac{a}{N_T}} - \arcsin\sqrt{\frac{b}{N_C}} \qquad \text{(Equation 1)}$$

The use of arcsine transformation can be dated back to the 1940s[18,19]. The main advantages of using ASD are that the variance of the point estimate (*i.e.*, ASD) is determined solely by the sample size and that it handles occurrences of 0 counts, allowing for incorporation of trials with 0 events in both control and treatment groups into meta-analyses[20].

Relative odds

Odds ratio (ORs) were calculated using the Yusuf-Peto method[21]. Although widely used, the Mantel-Haenszel method cannot include trials with zero events from either one or both groups without substituting zero with a non-zero number. The Yusuf-Peto method can include single-zero studies; thus, the Yusuf-Peto odds ratio (OR_{peto}) has been recognized as a relatively efficient estimator, especially when treatment effects from trials are not large or the sample size is similar between two groups[22]. The OR_{peto} can be calculated with Equation 2:

$$\text{Log}\widehat{OR}_{Peto} = (a - \frac{a+b}{n_T+n_C} * n_T)* \frac{(n_T+n_C)^2/(n_T+n_C-1)}{(a+b)(n_T-a+n_C-b)n_Tn_C} \qquad \text{(Equation 2)}$$

Where n_T, n_C, a and b denote the same as in Equation 1.

However, the Yusuf-Peto method cannot include double-zero studies. Existing approaches that have been used for meta-analysis on TNFα inhibitors are to exclude double-zero studies and, for single-zero studies, to change zero counts by adding either 0.5 or a similar number that is inversely proportional to the relative size of the opposite group[9,10,23]. These analytical treatments are not biologically supported. In the case of excluding double-zero studies, the results will bias away from the null hypothesis. Thus, we proposed an epidemiologically-based background correction (EBC). Our approach was to estimate the expected number of incidence cases (*e.g.*, if 0.01 TB cases were expected from an experimental arm, such TB cases could not be "observed" but using 0.01 to replace 0 would reflect the underlying epidemiology). Our correction assumed that the incidence of TB infection was 20 cases/100000 person-years as reported for patients with inflammatory bowel disease (IBD) in the United Kingdom[24]. EBC was then calculated according to Equation 3 below.

$$\text{EBC} = n \times \textit{Follow up duration (years)} \times \frac{20 \text{ cases}}{100,000 \text{ person-years}} \text{ (Equation 3)}$$

Where n is the number of subjects in either the treatment or the placebo group. The EBC was added into counts for both TB and non-TB for any trials reporting zero occurrences.

Statistical analysis

Statistical analysis followed the intent-to-treat principle. R version 3.4.3[25] with the "meta" package was used to

make plots and calculate the Yusuf-Peto odds ratio and ASD along with the corresponding confidence intervals (calculation accuracy was verified through manual calculation on a Microsoft Excel spreadsheet). Weight of contribution from individual studies to the pooled estimate was based on the inverse variance of the point estimate for individual studies. Inter-study variance was estimated using the DerSimonian-Laird method[26]. Two-sided *P* values of less than 0.05 and the 95% confidence interval (95%CI) excluding the null indicated statistical significance.

RESULTS

Search results
The selection process was summarized in Figure 1. A total of 748 articles were located from PubMed. Based on the review of titles and abstracts, 706 studies were not relevant or were duplicates, and they were subsequently excluded. The remaining 42 articles were more closely examined to determine inclusion in the analysis. Six studies were excluded because they were head-to-head, 3 were not placebo-controlled, 5 did not study EMA and FDA approved drugs, and 3 studies were duplicates (measured the same sample). Two trials were located through clinicaltrials.gov; of which, one (NCT00291668) did not post the results and was excluded. Thus, a total of 23 studies were included in the meta-analysis[27-48].

The 23 studies (Table 1) has evaluated adalimumab (7 studies; 1726 patients), certolizumab pegol (6 studies; 2,008 patients), and infliximab (10 studies; 1935 patients). Both induction and maintenance studies were included (5 induction; 18 maintenance). A total of 5669 patients underwent the clinical trials, with 3275 patients in the treatment group and 2394 patients in the control group. Follow-up duration ranged from 4 to 104 wk (mean follow-up duration = 32 wk). A total of about 3558 person-years were exposed to TNFα inhibitors or placebo (2033 person-years in the treatment, 1525 in person-years in the control). Publication dates ranged from 1997 to 2016. A total of 6 cases of TB were reported; all were from the anti-TNFα treatment groups. Two cases of TB were reported with adalimumab (one study), 1 with certolizumab pegol, and 3 with infliximab (three studies).

Risk difference
The risk difference between TNFα inhibitors and placebo was 0.028 (95%CI: 0.0011-0.055; *P* < 0.05) (Table 2). Random model results are presented, although the inter-study variance did not additionally contribute to the total variance of the pooled ASD (*i.e.*, the DerSimonian-Laird estimate of inter-study variance was zero). The weights for each drug - adalimumab, certolizumab pegol, and infliximab - were 28.9%, 36.9%, and 34.2% respectively. The respective risk differences were 0.028, 0.015, and 0.042 (Figure 2).

The funnel plot of the ASDs (Figure 3) indicates that trials showing no difference were apparently more likely published. This did not suggest publication bias, since risk of TB infection was neither the reason for publishing those trials nor the primary objective of those studies. In fact, the funnel plot reflects the fact that larger studies provided better chance to detect rare risks than smaller ones.

Relative odds
The sizes of the treatment arms were mostly similar to the sizes of the control arms, with the median ratio being 1.03. However, particular studies had very unbalanced arm sizes (maximum ratio = 3.32:1, average ratio = 1.64:1). The odds ratio was 4.85 (95%CI: 1.02-22.99; *P* < 0.05) with EBC and 5.85 (95%CI: 1.13-30.38; *P* < 0.05) without EBC (Table 2). The random effects model was not used because the Yusuf-Peto odds ratio was calculated under the assumption of a fixed effects model[21]. Weights for adalimumab, certolizumab pegol, and infliximab were 31.1%, 18.2%, and 50.7%, respectively, in the analysis with EBC (Figure 4). Without EBC, only 5 studies could be included (with the weight of 31.5% for adalimumab, 17.7% for certolizumab pegol, and 50.8% for infliximab).

Expected number of anti-TNFα treated cases to observe a tuberculosis case
If the background TB infection incidence in patients was 20 cases/100000 person-years, one TB case might be expected from a community of 5000 CD patients within a year. An ASD of 0.028 would be translated into a TB incidence of 177 cases/100000 person-years; thus, 1 TB case may be expected when treating 565 patients with TNFα inhibitors for one year (Table 3). If the harm effect of TNFα inhibitors can be described on a multiplicative scale as shown from the pooled Yusuf-Peto odds ratio, the numbers of patients to be treated to expect 1 TB case might be around 855 to 1031 (Table 3).

DISCUSSION
One of the most common complications following the use of TNFα inhibitors is increasing frequency of opportunistic infections[49]. Particularly, there is strong evidence linking mycobacterial infection to TNFα inhibitors, and TB infection risk is higher among patients receiving infliximab in comparison to controls[50]. Interestingly, TNFα-deficient animal models were more susceptible to mycobacterial infections compared to wild-type controls, although there was no survival rate difference in a healthy environment[51]. This indicates that TNFα plays a critical role in the immune response against mycobacterial infections.

Several studies have shown that there is a microbial factor affecting CD patients, and MAP was isolated from intestinal tissues, blood, and milk samples of not

Table 1 Summary of randomized, placebo-controlled, double-masked trials included

Study	Follow-up duration (wk)	Anti-TNFα treatment		Placebo	
		N[1]	N[2]	N[1]	N[2]
Adalimumab					
Hanauer *et al*[44], 2006	4	0	225	0	74
Colombel *et al*[47], 2007	52	2	517	0	261
Sandborn *et al*[35], 2007a	52	0	37	0	18
Sandborn *et al*[34], 2007b	4	0	159	0	166
Rutgeerts *et al*[37], 2012	48	0	64	0	65
Watanabe *et al*[28], 2012	52	0	25	0	25
Watanabe *et al*[28], 2012	4	0	67	0	23
		Total	1094		632
Certolizumab pegol					
Winter *et al*[27], 2004	12	0	66	0	24
Schreiber *et al*[30], 2005	20	0	145	0	73
Sandborn *et al*[36], 2007c	26	0	331	0	329
Schreiber *et al*[31], 2007	20	1	216	0	212
Sandborn *et al*[33], 2011	6	0	223	0	215
UCB Pharma[42], 2014	36	0	87	0	87
		Total	1068		940
Infliximab					
Targan *et al*[29], 1997	12	0	83	0	25
D'Haens *et al*[46], 1999	4	0	22	0	8
Present *et al*[41], 1999	18	0	63	0	31
Rutgeerts *et al*[38], 1999	36	0	37	0	36
Hanauer *et al*[45], 2002	44	1	385	0	188
Sands *et al*[32], 2004	40	0	139	0	143
Lémann *et al*[43], 2006	52	0	57	0	58
Colombel *et al*[48], 2010	30	1	169	0	170
Regueiro *et al*[40], 2011	52	0	11	0	13
Regueiro *et al*[39], 2016	104	1	147	0	150
		Total	1113		822

[1]Number of TB cases; [2]Number of total subjects.

Table 2 Risk of *Mycobacterium tuberculosis* infection associated with the use of tumor necrosis factor-alpha inhibitors in patients with Crohn's disease

	n	Risk estimate	95%CI	*P* value
Risk difference odds ratio	23	0.028	(0.0011, 0.055)	0.042
Including Double-Zero Studies	23	4.85	(1.02, 22.99)	0.047
Excluding Double-Zero Studies	5	5.85	(1.13, 30.38)	0.036

n: Number of individual clinical studies pooled together.

only CD patients but also patients with RA and type 1 diabetes[52-58]. Since MAP shares molecular homology and activity similar to TB, inducing TB infection susceptibility is an alarming sign for the immune response against MAP infection[58-60].

This study advances knowledge and awareness of the association between TNFα inhibitors and TB among CD patients. First, a non-biased estimation of TB infection risk associated with TNFα inhibitors for CD treatment was performed through arcsine transformation of TB incidence, which enabled the inclusion of all qualified studies including double-zero studies in the analysis. Second, a novel, epidemiologically-based background correction to adjust for zero counts was developed to enable the inclusion of double-zero studies into the estimation of the relative effect (odds ratio in this study). Lastly, with the use of these analytical approaches, a

significant increase of TB infection risk associated with using TNFα inhibitors to treat CD was shown from existing evidence, challenging findings of previous studies.

In our study, all 23 qualified trials were included. Among these 23 studies, 18 (78%) did not report TB cases from either the anti-TNFα treatment or the control group; these double-zero studies would have been excluded if we had followed the methods that previous meta-analyses in this area took. The double-zero observation was not a surprise. TB infection was rare in the Americas, Europe, Japan, Austria, and South Africa, where these RCTs were conducted. The median sample size of the control group across these 23 studies was 73 people; the median follow-up duration was 30 wk. Mathematically, only about 0.0084 TB cases would be expected in these control patients if the background TB

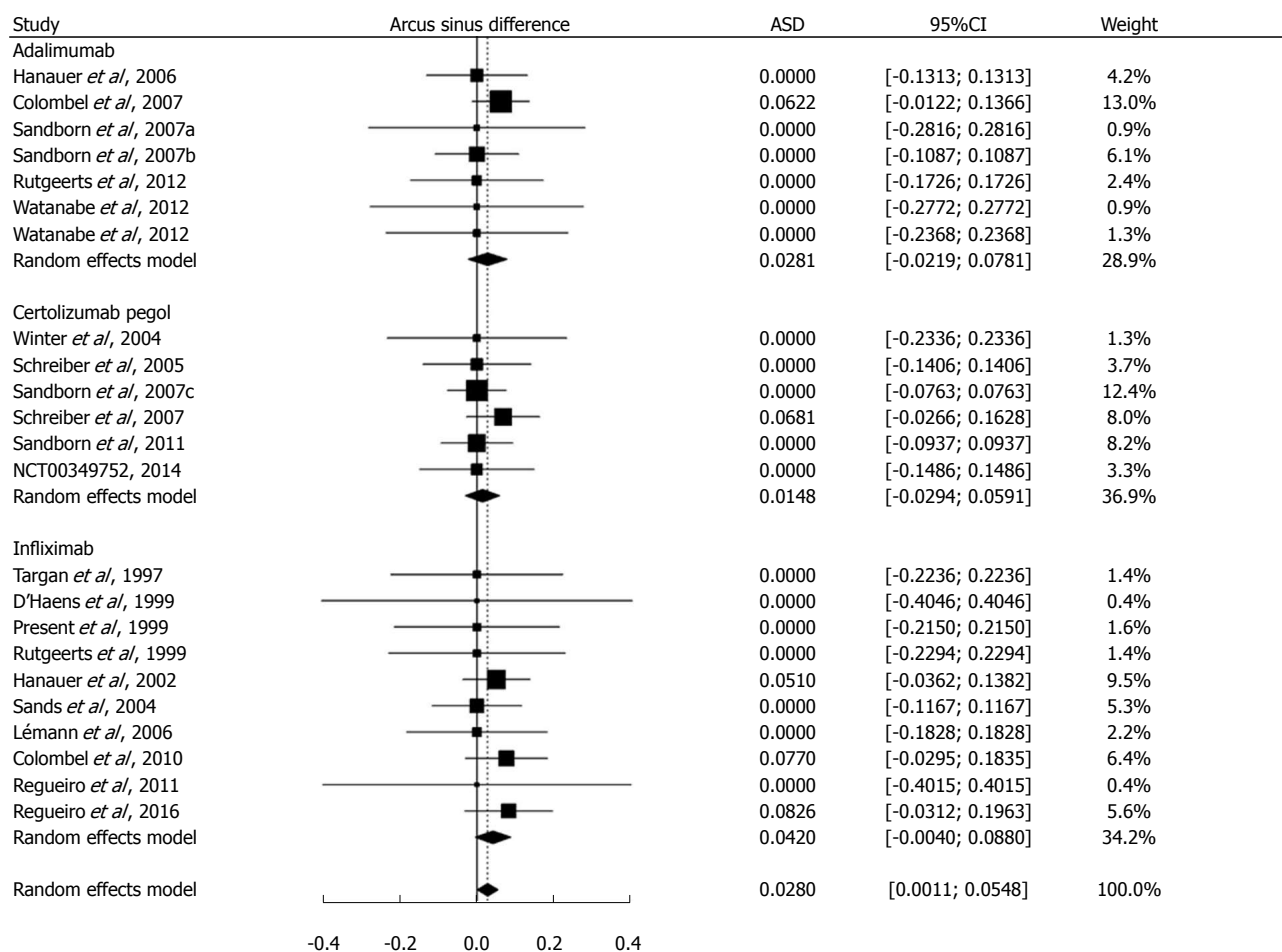**Table 3 Estimated absolute incidence of *Mycobacterium tuberculosis* infection in patients with Crohn's disease treated with tumor necrosis factor-alpha inhibitors and the number of patients with Crohn's disease who need to be treated with tumor necrosis factor-alpha inhibitors to expect one *Mycobacterium tuberculosis* case**

	Incidence of TB with TNFα treatment (cases/person-years)[1]	Number of patients treated to see one TB Case in one year
Based on risk difference	177/100000	565
Based on relative odds estimated with background correction	97/100000	1031
Based on relative odds estimated without background correction	117/100000	855

[1]The background incidence was assumed to be 20 cases/100000 person-years as reported by Aberra *et al*[24]. TB: *Mycobacterium tuberculosis*.

Figure 1 Evidence collection and selection.

infection incidence was 20 cases/100000 person-years as reported by Aberra *et al*[24]. If TNFα inhibitors had increased the TB infection risk by 5 times, there might have been about a 4% chance to observe 1 TB case in the anti-TNFα treatment arm. Meta-analysis provides excellent opportunities to pool multiple studies together to improve the estimation of the chance of observing a TB case. Discarding these double-zero studies (78% of the studies in our analysis) might decrease the value of meta-analysis.

We regarded the risk difference calculated after arcsine transformation of incidence as the primary results. The ASD method does not call for any correction for zero counts. Additionally, the robustness of an ASD estimate is not contingent on the effect size and the treatment-to-control balance of sample size. These analytical features provided a distinct advantage over either the Yusuf-Peto method or the Mantel-Haenszel method. However, from a biological perspective, it is

possible that the TB infection risk from the use of TNFα inhibitors in patients with CD may be better described on a multiplicative scale (relative scale). Thus, a risk difference of 0.028 could be translated into increasing the risk of TB by a factor of 8, as the number needed to harm was calculated at 565 patients compared to the background number needed to harm of 5000 patients (Table 3). However, the 8 times increased risk of TB (based off ASD) only applies if the incidence of TB remains constant (20 cases/100000 person-years). We thus performed further analysis to better describe this multiplicative scale, for which we chose the Yusuf-Peto method because, as compared to the Mantel-Haenszel method, it can handle single-zero studies[61]. Unfortunately, the Yusuf-Peto method cannot handle double-zero studies.

We proposed an epidemiologically-based background correction (*i.e.*, EBC) to mathematically replace zeros. If metrics other than ASD (*e.g.*, odds ratio, hazard ratio or

Study	Arcus sinus difference	ASD	95%CI	Weight
Adalimumab				
Hanauer *et al*, 2006		0.0000	[-0.1313; 0.1313]	4.2%
Colombel *et al*, 2007		0.0622	[-0.0122; 0.1366]	13.0%
Sandborn *et al*, 2007a		0.0000	[-0.2816; 0.2816]	0.9%
Sandborn *et al*, 2007b		0.0000	[-0.1087; 0.1087]	6.1%
Rutgeerts *et al*, 2012		0.0000	[-0.1726; 0.1726]	2.4%
Watanabe *et al*, 2012		0.0000	[-0.2772; 0.2772]	0.9%
Watanabe *et al*, 2012		0.0000	[-0.2368; 0.2368]	1.3%
Random effects model		0.0281	[-0.0219; 0.0781]	28.9%
Certolizumab pegol				
Winter *et al*, 2004		0.0000	[-0.2336; 0.2336]	1.3%
Schreiber *et al*, 2005		0.0000	[-0.1406; 0.1406]	3.7%
Sandborn *et al*, 2007c		0.0000	[-0.0763; 0.0763]	12.4%
Schreiber *et al*, 2007		0.0681	[-0.0266; 0.1628]	8.0%
Sandborn *et al*, 2011		0.0000	[-0.0937; 0.0937]	8.2%
NCT00349752, 2014		0.0000	[-0.1486; 0.1486]	3.3%
Random effects model		0.0148	[-0.0294; 0.0591]	36.9%
Infliximab				
Targan *et al*, 1997		0.0000	[-0.2236; 0.2236]	1.4%
D'Haens *et al*, 1999		0.0000	[-0.4046; 0.4046]	0.4%
Present *et al*, 1999		0.0000	[-0.2150; 0.2150]	1.6%
Rutgeerts *et al*, 1999		0.0000	[-0.2294; 0.2294]	1.4%
Hanauer *et al*, 2002		0.0510	[-0.0362; 0.1382]	9.5%
Sands *et al*, 2004		0.0000	[-0.1167; 0.1167]	5.3%
Lémann *et al*, 2006		0.0000	[-0.1828; 0.1828]	2.2%
Colombel *et al*, 2010		0.0770	[-0.0295; 0.1835]	6.4%
Regueiro *et al*, 2011		0.0000	[-0.4015; 0.4015]	0.4%
Regueiro *et al*, 2016		0.0826	[-0.0312; 0.1963]	5.6%
Random effects model		0.0420	[-0.0040; 0.0880]	34.2%
Random effects model		0.0280	[0.0011; 0.0548]	100.0%

Figure 2 Difference of the risk of *Mycobacterium tuberculosis* infection between patients with Crohn's disease treated with tumor necrosis factor-alpha inhibitors and those treated with placebo. Risk difference was calculated after arcsine transformation of *Mycobacterium tuberculosis* incidence (Arcus sinus difference, ASD) and indicated by the numbers on x-axis. Weight: the percentage contribution of an individual study to the overall estimation. The x-axis indicates the risk difference. The vertical dashed line indicates the overall point estimate. The solid horizontal lines show the confidence interval (CI). The size of the black box and diamond is proportional to the corresponding weight.

Figure 3 Relationship between the estimated *Mycobacterium tuberculosis* infection risk difference and the corresponding standard error of the estimate. Risk difference was calculated after arcsine transformation of *Mycobacterium tuberculosis* incidence (Arcus sinus difference, ASD). The dashed vertical line indicates the overall arcsine difference found, and the diagonal lines indicate the expected 95% confidence intervals associated with the expected mean ASD for clinical trials with various numbers of study subjects.

rate ratio) have to be estimated and the risk of interest is so rare that even one occurrence is not expected, we recommend that EBC be used for continuity correction instead of adding 0.5 (or a similar number depending on the ratio of sample size between treatment and control groups) or statistical-model based estimates[62,63]. The latter approaches lack biological considerations, and in the case of adding a number around 0.5, artificially make a much larger background incidence than there actually is (it would have boosted the background incidence by ~60 times in this meta-analysis).

There are major limitations with the use of EBC. The EBC was based off TB incidence rate in the UK IBD populations. Although the RCTs in this study were largely conducted in Western countries, the TB incidence of Crohn's patients in the UK may not represent the TB incidence of the countries in which the clinical trials were conducted, let alone the patients who participated in the clinical trial. Furthermore, the TB incidence rate was found in populations with IBD, which may not be

Study	Anti-TNF		Control		Odds ratio	OR	Weight
	TB cases	Total	TB cases	Total			
Adalimumab							
Hanauer *et al*, 2006	0.0035	225	0.0011	74		1.05	0.054%
Colombel *et al*, 2007	2.1031	517	0.0521	261		4.05	30.276%
Sandborn *et al*, 2007a	0.0074	37	0.0036	18		1.00	0.156%
Sandborn *et al*, 2007b	0.0024	159	0.0025	166		1.00	0.078%
Rutgeerts *et al*, 2012	0.0118	64	0.0120	65		1.00	0.378%
Watanabe *et al*, 2012	0.0050	25	0.0050	25		1.00	0.161%
Watanabe *et al*, 2012	0.0010	67	0.0004	23		0.85	0.017%
Fixed effects model		1094		632		3.90	31.119%
Certolizumab pegol							
Winter *et al*, 2004	0.0030	66	0.0011	24		0.99	0.051%
Schreiber *et al*, 2005	0.0111	145	0.0056	73		1.00	0.236%
Sandborn *et al*, 2007c	0.0330	331	0.0328	329		1.00	1.040%
Schreiber *et al*, 2007	1.0166	216	0.0163	212		6.81	16.293%
Sandborn *et al*, 2011	0.0051	223	0.0049	215		1.00	0.158%
NCT00349752, 2014	0.0120	87	0.0120	87		1.00	0.381%
Fixed effects model		1068		940		5.59	18.159%
Infliximab							
Targan *et al*, 1997	0.0038	83	0.0012	25		0.95	0.057%
D'Haens *et al*, 1999	0.0003	22	0.0001	8		1.09	0.005%
Present *et al*, 1999	0.0043	63	0.0021	31		1.01	0.090%
Rutgeerts *et al*, 1999	0.0051	37	0.0050	36		0.99	0.162%
Hanauer *et al*, 2002	1.0650	385	0.0317	188		3.89	15.255%
Sands *et al*, 2004	0.0213	139	0.0219	143		1.00	0.684%
Lémann *et al*, 2006	0.0114	57	0.0116	58		1.00	0.366%
Colombel *et al*, 2010	1.0194	169	0.0196	170		6.89	16.390%
Regueiro *et al*, 2011	0.0022	11	0.0026	13		1.00	0.078%
Regueiro *et al*, 2016	1.0586	147	0.0598	150		6.09	17.636%
Fixed effects model		1113		822		5.26	50.722%
Fixed effects model		3275		2394		4.85	100.000%

0.01   0.1   0.5   2   10   400

Figure 4 Odds of patients developing active *Mycobacterium tuberculosis* when treated with tumor necrosis factor-alpha inhibitors for Crohn's disease relative to those treated with placebo. Odds ratio (OR) was calculated using the Yusuf-Peto method and indicated by the numbers on the x-axis. Number of *Mycobacterium tuberculosis* cases was modified using the background *Mycobacterium tuberculosis* incidence, *i.e.*, adding to the reported number of *Mycobacterium tuberculosis* cases with a number (less than 1) that might be expected from a given number of patients (listed under "Total"). Weight: the percentage contribution of an individual study to the pooled estimate. The vertical dashed line indicates the pooled odds ratio. The solid horizontal lines show the confidence interval (CI). The size of the black box and diamond is proportional to the corresponding weight. *Mycobacterium tuberculosis* here denotes tuberculosis.

representative of TB incidence in the population with CD.

Aside from the analytical approach to avoid Simpson's paradox, the validity to pool results from individual studies in this meta-analysis largely resides in the fact that each study had a placebo-treatment arm. The impact from the difference of study populations was therefore minimized, as the end point (risk difference or odds ratio) mainly reflected the effect of TNFα inhibitors [the effect of confounders was either subtracted out (for ASD) or normalized (for ORs)]. Thus, common factors restricting the use of meta-analysis, such as geographic location, population characteristics, exposure, maintenance *vs* induction trials, status (*e.g.*, phase 3 *vs* phase 4) of the clinical trials, secular trend, and TB screening methods could be assumed to be not of major concern.

Perhaps the fact that only studies in English were included in the analysis may limit the generalizability of the results, considering that the demographics and trends of CD and TB infection differ among different

regions or different populations[64]. The included studies were mainly EMA- and FDA-regulated clinical trials conducted in western countries. In fact, only one study that contained strictly Asian populations was included in this meta-analysis[28]. EBC may also be compromised, as the prevalence and incidence of TB infection was higher in Asian countries[65]. Thus, caution should be taken when extrapolating the results from this analysis to predict TB infection risk of TNFα inhibitors treating CD in non-western countries.

Additional attention should be paid to TB screening. Patients could have had either latent TB infection that was reactivated or acquired TB infection through exposure. The screening methods of trials varied and often went unreported. Furthermore, screening out patients based on a positive tuberculin skin test may have different impacts on the TB infection occurrence due to the different practices of Bacillus Calmette-Guérin vaccinations[66]. Lastly, two studies did not report screening methods[39,45]. Close examination of the additional details of TB screening may provide further

insight on the nature of TB infection - whether it was acquired or reactivated.

We conclude that there is sufficient evidence to assert that using TNFα inhibitors increases the risk of developing TB infection in patients with CD. Twenty-three studies were analyzed, and multiple statistical methods repeatedly gave significant risk. To our knowledge, these 23 studies represented all appropriate literature available for the topic at hand, with an extensive and careful review conducted. No studies were excluded, provided that they used a placebo control and were randomized and masked. The randomization minimized potential confounding such as age, duration of IBD, and disease activity. These results challenge findings of previous studies, which reported no significantly increased risk of TB infection when TNFα inhibitors were used to manage patients with CD[10,14]. Based on the risk difference found in this study, on average 565 patients treated with TNFα inhibitors may result in 1 patient getting infected with TB, *vs* 5000 patients not treated with TNFα inhibitors producing 1 case of TB, if the background incidence of TB infection in moderately severe CD is similar to the rates found in the UK IBD population.

The etiology of CD remains uncertain. Evidence suggests that CD may be caused by an immune response to commensal enteric bacteria[67]. Recent research also suggests that CD is intimately linked to MAP, which is a TB-like bacterium[11,55,57]. The use of TNFα inhibitors in these patients could favor MAP infection and worsen the patient condition. It is currently difficult to come to conclusions considering that the RCTs did not test for MAP infection - much less reported it. Further research could be done on looking at patient outcomes and determining which patients had MAP infection and what their susceptibility to infection was.

ARTICLE HIGHLIGHTS

Research background
Recent literature has identified many adverse effects from the use of tumor necrosis factor alpha (TNFα) inhibitors. Among these are an increased risk of opportunistic infections and serious adverse events. However, previous meta-analyses have not identified a significantly increased risk of tuberculosis (TB) infection, which is especially pertinent considering that *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is suspected to have an intimate role in the etiology of Crohn's disease (CD).

Research motivation
Due to the suspected role of MAP in the etiology of CD and previous literature on the topic, TNFα inhibitors likely increase the risk of TB infection, which would guide future clinical decisions. However, such an association has not been adequately quantified. Additionally, current statistical models commonly used in meta-analyses fail to adequately analyze data where events are rare (defined as less than 1 case per 1000 person-years). Our research would not only bring additional considerations when making clinical decisions about anti-TNFα therapy but also introduce existing statistical models and novel corrections that can help deal with rare events.

Research objectives
In this study, we seek to advance the awareness of and quantify the association between TNFα inhibitors and TB in CD patients. We seek to include all qualified studies - including studies with zero events in the treatment and control groups - without using corrections, which previous meta-analyses have failed to do. Finally, we seek to introduce a novel, epidemiologically-based background correction (EBC) that can adjust for zero counts.

Research methods
The Preferred Reporting Items for the Systematic reviews and Meta-Analyses (PRISMA) protocol was followed. Only randomized, placebo-controlled trials (RCTs) were considered. Arcsine differences were used to calculate risk differences in a non-biased way. Odds ratios were calculated using the Yusuf-Peto method both with and without corrections (EBC).

Research results
Twenty-three RCTs were analyzed, and all the statistical methods repeatedly provided significantly increased risk of TB infection. A risk difference (RD) of 0.028 (95%CI: 0.0011-0.055) was calculated. The odds ratio (OR) was 4.85 (95%CI: 1.02-22.99) when all studies were included using EBCs and 5.85 (95%CI: 1.13-30.38) when studies reporting zero tuberculosis cases were excluded.

Research conclusions
There is an increased risk of TB infection in patients with Crohn's disease who use TNFα inhibitors. This risk could range from 5 times (OR) to as high as 8 times (RD). Alternative therapy such as using more antibiotics and less immunosuppressive agents may be evaluated.

Research perspectives
This study provided us with additional approaches that can be considered when conducting future meta-analyses. ASD is a particularly useful method that can contribute to future meta-analyses. The relationship between MAP and TB is still unclear. Further research on the validity of the EBC should be pursued.

REFERENCES

1   **Molodecky NA**, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 2012; **142**: 46-54.e42; quiz e30 [PMID: 22001864 DOI: 10.1053/j.gastro.2011.10.001]

2   **Qasem A**, Naser AE, Naser SA. The alternate effects of anti-TNFα therapeutics and their role in mycobacterial granulomatous infection in Crohn's disease. *Expert Rev Anti Infect Ther* 2017; **15**: 637-643 [PMID: 28481651 DOI: 10.1080/14787210.2017.1328276]

3   **Braegger CP**, Nicholls S, Murch SH, Stephens S, MacDonald TT. Tumour necrosis factor alpha in stool as a marker of intestinal inflammation. *Lancet* 1992; **339**: 89-91 [PMID: 1345871]

4   **Swaminath A**, Lebwohl B, Capiak KM, Present DH. Practice patterns in the use of anti-tumor necrosis factor alpha agents in the management of Crohn's disease: a US national practice survey comparing experts and non-experts. *Dig Dis Sci* 2011; **56**: 1160-1164 [PMID: 21181440 DOI: 10.1007/s10620-010-1530-9]

5   **Roda G**, Jharap B, Neeraj N, Colombel JF. Loss of Response to Anti-TNFs: Definition, Epidemiology, and Management. *Clin Transl Gastroenterol* 2016; **7**: e135 [PMID: 26741065 DOI: 10.1038/ctg.2015.63]

6   **Kalden JR**, Schulze-Koops H. Immunogenicity and loss of response to TNF inhibitors: implications for rheumatoid arthritis treatment. *Nat Rev Rheumatol* 2017; **13**: 707-718 [PMID: 29158574 DOI: 10.1038/nrrheum.2017.187]

7   **Hyrich KL**, Watson KD, Silman AJ, Symmons DP; British Society for Rheumatology Biologics Register. Predictors of response to anti-TNF-alpha therapy among patients with rheumatoid arthritis: results from the British Society for Rheumatology Biologics Register. *Rheumatology* (Oxford) 2006; **45**: 1558-1565 [PMID: 16705046 DOI: 10.1093/rheumatology/kel149]

8   **Gisbert JP**, Marín AC, Chaparro M. The Risk of Relapse after Anti-TNF Discontinuation in Inflammatory Bowel Disease: Systematic Review and Meta-Analysis. *Am J Gastroenterol* 2016; **111**: 632-647 [PMID: 27002797 DOI: 10.1038/ajg.2016.54]

9   **Minozzi S**, Bonovas S, Lytras T, Pecoraro V, González-Lorenzo M, Bastiampillai AJ, Gabrielli EM, Lonati AC, Moja L, Cinquini M, Marino V, Matucci A, Milano GM, Tocci G, Scarpa R, Goletti D, Cantini F. Risk of infections using anti-TNF agents in rheumatoid arthritis, psoriatic arthritis, and ankylosing spondylitis: a systematic review and meta-analysis. *Expert Opin Drug Saf* 2016; **15**: 11-34 [PMID: 27924643 DOI: 10.1080/14740338.2016.1240783]

10   **Bonovas S**, Fiorino G, Allocca M, Lytras T, Nikolopoulos GK, Peyrin-Biroulet L, Danese S. Biologic Therapies and Risk of Infection and Malignancy in Patients With Inflammatory Bowel Disease: A Systematic Review and Network Meta-analysis. *Clin Gastroenterol Hepatol* 2016; **14**: 1385-1397.e10 [PMID: 27189910 DOI: 10.1016/j.cgh.2016.04.039]

11   **Naser SA**, Sagramsingh SR, Naser AS, Thanigachalam S. Mycobacterium avium subspecies paratuberculosis causes Crohn's disease in some inflammatory bowel disease patients. *World J Gastroenterol* 2014; **20**: 7403-7415 [PMID: 24966610 DOI: 10.3748/wjg.v20.i23.7403]

12   **Roach DR**, Bean AG, Demangel C, France MP, Briscoe H, Britton WJ. TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. *J Immunol* 2002; **168**: 4620-4627 [PMID: 11971010]

13   **Williams CJ**, Peyrin-Biroulet L, Ford AC. Systematic review with meta-analysis: malignancies with anti-tumour necrosis factor-α therapy in inflammatory bowel disease. *Aliment Pharmacol Ther* 2014; **39**: 447-458 [PMID: 24444171 DOI: 10.1111/apt.12624]

14   **Ford AC**, Peyrin-Biroulet L. Opportunistic infections with anti-tumor necrosis factor-α therapy in inflammatory bowel disease: meta-analysis of randomized controlled trials. *Am J Gastroenterol* 2013; **108**: 1268-1276 [PMID: 23649185 DOI: 10.1038/ajg.2013.138]

15   **Qasem A**, Safavikhasraghi M, Naser SA. A single capsule formulation of RHB-104 demonstrates higher anti-microbial growth potency for effective treatment of Crohn's disease associated with Mycobacterium avium subspecies paratuberculosis. *Gut Pathog* 2016; **8**: 45 [PMID: 27708718 DOI: 10.1186/s13099-016-0127-z]

16   **Cao B,** Naser SA. Systematic review and meta-analysis on the risk of tuberculosis in patients with Crohn's disease who take TNFα inhibitors. PROSPERO: International prospective register of systematic reviews, 2018

17   **Moher D**, Liberati A, Tetzlaff J, Altman DG; PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *BMJ* 2009; **339**: b2535 [PMID: 19622551 DOI: 10.1136/bmj.b2535]

18   **Freeman M**, Tukey J. Transformations related to the angular and the square root. *Annals of Mathematical Statistics* 1950; **21**: 607-611

19   **Anscombe FJ**. The transformation of Poisson, binomial and negative-binomial data. *Biometrika* 1948; **35**: 246-254 [DOI: 10.2307/2332343]

20   **Rücker G**, Schwarzer G, Carpenter J, Olkin I. Why add anything to nothing? The arcsine difference as a measure of treatment effect in meta-analysis with zero cells. *Stat Med* 2009; **28**: 721-738 [PMID: 19072749 DOI: 10.1002/sim.3511]

21   **Yusuf S**, Peto R, Lewis J, Collins R, Sleight P. Beta blockade during and after myocardial infarction: an overview of the randomized trials. *Prog Cardiovasc Dis* 1985; **27**: 335-371 [PMID: 2858114]

22   **Brockhaus AC**, Bender R, Skipka G. The Peto odds ratio viewed as a new effect measure. *Stat Med* 2014; **33**: 4861-4874 [PMID:

23   **Bongartz T**, Sutton AJ, Sweeting MJ, Buchan I, Matteson EL, Montori V. Anti-TNF antibody therapy in rheumatoid arthritis and the risk of serious infections and malignancies: systematic review and meta-analysis of rare harmful effects in randomized controlled trials. *JAMA* 2006; **295**: 2275-2285 [PMID: 16705109 DOI: 10.1001/jama.295.19.2275]

24   **Aberra FN**, Stettler N, Brensinger C, Lichtenstein GR, Lewis JD. Risk for active tuberculosis in inflammatory bowel disease patients. *Clin Gastroenterol Hepatol* 2007; **5**: 1070-1075 [PMID: 17627901 DOI: 10.1016/j.cgh.2007.04.007]

25   **R Core Team**. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available from: URL: http://www.r-project.org/

26   **DerSimonian R**, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986; **7**: 177-188 [PMID: 3802833]

27   **Winter TA**, Wright J, Ghosh S, Jahnsen J, Innes A, Round P. Intravenous CDP870, a PEGylated Fab' fragment of a humanized antitumour necrosis factor antibody, in patients with moderate-to-severe Crohn's disease: an exploratory study. *Aliment Pharmacol Ther* 2004; **20**: 1337-1346 [PMID: 15606396 DOI: 10.1111/j.1365-2036.2004.02285.x]

28   **Watanabe M**, Hibi T, Lomax KG, Paulson SK, Chao J, Alam MS, Camez A; Study Investigators. Adalimumab for the induction and maintenance of clinical remission in Japanese patients with Crohn's disease. *J Crohns Colitis* 2012; **6**: 160-173 [PMID: 22325170 DOI: 10.1016/j.crohns.2011.07.013]

29   **Targan SR**, Hanauer SB, van Deventer SJ, Mayer L, Present DH, Braakman T, DeWoody KL, Schaible TF, Rutgeerts PJ. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N Engl J Med* 1997; **337**: 1029-1035 [PMID: 9321530 DOI: 10.1056/nejm199710093371502]

30   **Schreiber S**, Rutgeerts P, Fedorak RN, Khaliq-Kareemi M, Kamm MA, Boivin M, Bernstein CN, Staun M, Thomsen OØ, Innes A; CDP870 Crohn's Disease Study Group. A randomized, placebo-controlled trial of certolizumab pegol (CDP870) for treatment of Crohn's disease. *Gastroenterology* 2005; **129**: 807-818 [PMID: 16143120 DOI: 10.1053/j.gastro.2005.06.064]

31   **Schreiber S**, Khaliq-Kareemi M, Lawrance IC, Thomsen OØ, Hanauer SB, McColm J, Bloomfield R, Sandborn WJ; PRECISE 2 Study Investigators. Maintenance therapy with certolizumab pegol for Crohn's disease. *N Engl J Med* 2007; **357**: 239-250 [PMID: 17634459 DOI: 10.1056/NEJMoa062897]

32   **Sands BE**, Anderson FH, Bernstein CN, Chey WY, Feagan BG, Fedorak RN, Kamm MA, Korzenik JR, Lashner BA, Onken JE, Rachmilewitz D, Rutgeerts P, Wild G, Wolf DC, Marsters PA, Travers SB, Blank MA, van Deventer SJ. Infliximab maintenance therapy for fistulizing Crohn's disease. *N Engl J Med* 2004; **350**: 876-885 [PMID: 14985485 DOI: 10.1056/NEJMoa030815]

33   **Sandborn WJ**, Schreiber S, Feagan BG, Rutgeerts P, Younes ZH, Bloomfield R, Coteur G, Guzman JP, D'Haens GR. Certolizumab pegol for active Crohn's disease: a placebo-controlled, randomized trial. *Clin Gastroenterol Hepatol* 2011; **9**: 670-678.e3 [PMID: 21642014 DOI: 10.1016/j.cgh.2011.04.031]

34   **Sandborn WJ**, Rutgeerts P, Enns R, Hanauer SB, Colombel JF, Panaccione R, D'Haens G, Li J, Rosenfeld MR, Kent JD, Pollack PF. Adalimumab induction therapy for Crohn disease previously treated with infliximab: a randomized trial. *Ann Intern Med* 2007; **146**: 829-838 [PMID: 17470824]

35   **Sandborn WJ**, Hanauer SB, Rutgeerts P, Fedorak RN, Lukas M, MacIntosh DG, Panaccione R, Wolf D, Kent JD, Bittle B, Li J, Pollack PF. Adalimumab for maintenance treatment of Crohn's disease: results of the CLASSIC II trial. *Gut* 2007; **56**: 1232-1239 [PMID: 17299059 DOI: 10.1136/gut.2006.106781]

36   **Sandborn WJ**, Feagan BG, Stoinov S, Honiball PJ, Rutgeerts P, Mason D, Bloomfield R, Schreiber S; PRECISE 1 Study Investigators. Certolizumab pegol for the treatment of Crohn's disease. *N Engl J Med* 2007; **357**: 228-238 [PMID: 17634458 DOI:

10.1056/NEJMoa067594]

37   **Rutgeerts P**, Van Assche G, Sandborn WJ, Wolf DC, Geboes K, Colombel JF, Reinisch W; EXTEND Investigators, Kumar A, Lazar A, Camez A, Lomax KG, Pollack PF, D'Haens G. Adalimumab induces and maintains mucosal healing in patients with Crohn's disease: data from the EXTEND trial. *Gastroenterology* 2012; **142**: 1102-1111.e2 [PMID: 22326435 DOI: 10.1053/j.gastro.2012.01.035]

38   **Rutgeerts P**, D'Haens G, Targan S, Vasiliauskas E, Hanauer SB, Present DH, Mayer L, Van Hogezand RA, Braakman T, DeWoody KL, Schaible TF, Van Deventer SJ. Efficacy and safety of retreatment with anti-tumor necrosis factor antibody (infliximab) to maintain remission in Crohn's disease. *Gastroenterology* 1999; **117**: 761-769 [PMID: 10500056]

39   **Regueiro M**, Feagan BG, Zou B, Johanns J, Blank MA, Chevrier M, Plevy S, Popp J, Cornillie FJ, Lukas M, Danese S, Gionchetti P, Hanauer SB, Reinisch W, Sandborn WJ, Sorrentino D, Rutgeerts P; PREVENT Study Group. Infliximab Reduces Endoscopic, but Not Clinical, Recurrence of Crohn's Disease After Ileocolonic Resection. *Gastroenterology* 2016; **150**: 1568-1578 [PMID: 26946343 DOI: 10.1053/j.gastro.2016.02.072]

40   **Regueiro M**, El-Hachem S, Kip KE, Schraut W, Baidoo L, Watson A, Swoger J, Schwartz M, Barrie A, Pesci M, Binion D. Postoperative infliximab is not associated with an increase in adverse events in Crohn's disease. *Dig Dis Sci* 2011; **56**: 3610-3615 [PMID: 21681507 DOI: 10.1007/s10620-011-1785-9]

41   **Present DH**, Rutgeerts P, Targan S, Hanauer SB, Mayer L, van Hogezand RA, Podolsky DK, Sands BE, Braakman T, DeWoody KL, Schaible TF, van Deventer SJ. Infliximab for the treatment of fistulas in patients with Crohn's disease. *N Engl J Med* 1999; **340**: 1398-1405 [PMID: 10228190 DOI: 10.1056/ nejm199905063401804]

42   **UCB Pharma**. Corticosteroid Sparing Effect of Certolizumab in Crohn's Disease (COSPAR1) (NCT00349752; Last Update Posted on September 5, 2014). Available at ClinicalTrials.gov. Accessed on March 27, 2018.

43   **Lémann M**, Mary JY, Duclos B, Veyrac M, Dupas JL, Delchier JC, Laharie D, Moreau J, Cadiot G, Picon L, Bourreille A, Sobahni I, Colombel JF; Groupe d'Etude Therapeutique des Affections Inflammatoires du Tube Digestif (GETAID). Infliximab plus azathioprine for steroid-dependent Crohn's disease patients: a randomized placebo-controlled trial. *Gastroenterology* 2006; **130**: 1054-1061 [PMID: 16618399 DOI: 10.1053/j.gastro.2006.02.014]

44   **Hanauer SB**, Sandborn WJ, Rutgeerts P, Fedorak RN, Lukas M, MacIntosh D, Panaccione R, Wolf D, Pollack P. Human anti-tumor necrosis factor monoclonal antibody (adalimumab) in Crohn's disease: the CLASSIC-I trial. *Gastroenterology* 2006; **130**: 323-333; quiz 591 [PMID: 16472588 DOI: 10.1053/j.gastro.2005.11.030]

45   **Hanauer SB**, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, Rutgeerts P; ACCENT I Study Group. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* 2002; **359**: 1541-1549 [PMID: 12047962 DOI: 10.1016/ s0140-6736(02)08512-4]

46   **D'haens G**, Van Deventer S, Van Hogezand R, Chalmers D, Kothe C, Baert F, Braakman T, Schaible T, Geboes K, Rutgeerts P. Endoscopic and histological healing with infliximab anti-tumor necrosis factor antibodies in Crohn's disease: A European multicenter trial. *Gastroenterology* 1999; **116**: 1029-1034 [PMID: 10220494]

47   **Colombel JF**, Sandborn WJ, Rutgeerts P, Enns R, Hanauer SB, Panaccione R, Schreiber S, Byczkowski D, Li J, Kent JD, Pollack PF. Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial. *Gastroenterology* 2007; **132**: 52-65 [PMID: 17241859 DOI: 10.1053/j.gastro.2006.11.041]

48   **Colombel JF**, Sandborn WJ, Reinisch W, Mantzaris GJ, Kornbluth A, Rachmilewitz D, Lichtiger S, D'Haens G, Diamond RH, Broussard DL, Tang KL, van der Woude CJ, Rutgeerts P; SONIC

Study Group. Infliximab, azathioprine, or combination therapy for Crohn's disease. *N Engl J Med* 2010; **362**: 1383-1395 [PMID: 20393175 DOI: 10.1056/NEJMoa0904492]

49   **Antoni C**, Braun J. Side effects of anti-TNF therapy: current knowledge. *Clin Exp Rheumatol* 2002; **20**: S152-S157 [PMID: 12463468]

50   **Keane J**, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, Siegel JN, Braun MM. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med* 2001; **345**: 1098-1104 [PMID: 11596589 DOI: 10.1056/NEJMoa011110]

51   **Keane J**, Gershon SK, Braun MM. Tuberculosis and treatment with infliximab. *The New England journal of medicine* 2002; **346**: 2

52   **Schwartz D**, Shafran I, Romero C, Piromalli C, Biggerstaff J, Naser N, Chamberlin W, Naser SA. Use of short-term culture for identification of Mycobacterium avium subsp. paratuberculosis in tissue from Crohn's disease patients. *Clin Microbiol Infect* 2000; **6**: 303-307 [PMID: 11168138]

53   **Qasem A**, Abdel-Aty A, Abu-Suwa H, Naser SA. Oxidative stress due to Mycobacterium avium subspecies paratuberculosis (MAP) infection upregulates selenium-dependent GPx activity. *Gut Pathog* 2016; **8**: 12 [PMID: 26997979 DOI: 10.1186/s13099-016-0090-8]

54   **Naser SA**, Schwartz D, Shafran I. Isolation of Mycobacterium avium subsp paratuberculosis from breast milk of Crohn's disease patients. *Am J Gastroenterol* 2000; **95**: 1094-1095 [PMID: 10763975 DOI: 10.1111/j.1572-0241.2000.01954.x]

55   **Sharp RC**, Beg SA, Naser SA. Role of PTPN2/22 polymorphisms in pathophysiology of Crohn's disease. *World J Gastroenterol* 2018; **24**: 657-670 [PMID: 29456405 DOI: 10.3748/wjg.v24.i6.657]

56   **Sharp RC**, Beg SA, Naser SA. Polymorphisms in Protein Tyrosine Phosphatase Non-receptor Type 2 and 22 (PTPN2/22) Are Linked to Hyper-Proliferative T-Cells and Susceptibility to Mycobacteria in Rheumatoid Arthritis. *Front Cell Infect Microbiol* 2018; **8**: 11 [PMID: 29423382 DOI: 10.3389/fcimb.2018.00011]

57   **Sharp RC**, Abdulrahim M, Naser ES, Naser SA. Genetic Variations of PTPN2 and PTPN22: Role in the Pathogenesis of Type 1 Diabetes and Crohn's Disease. *Front Cell Infect Microbiol* 2015; **5**: 95 [PMID: 26734582 DOI: 10.3389/fcimb.2015.00095]

58   **Naser SA**, Thanigachalam S, Dow CT, Collins MT. Exploring the role of Mycobacterium avium subspecies paratuberculosis in the pathogenesis of type 1 diabetes mellitus: a pilot study. *Gut Pathog* 2013; **5**: 14 [PMID: 23759115 DOI: 10.1186/1757-4749-5-14]

59   **Bach H**, Papavinasasundaram KG, Wong D, Hmama Z, Av-Gay Y. Mycobacterium tuberculosis virulence is mediated by PtpA dephosphorylation of human vacuolar protein sorting 33B. *Cell Host Microbe* 2008; **3**: 316-322 [PMID: 18474358 DOI: 10.1016/ j.chom.2008.03.008]

60   **Bach H**, Ko HH, Raizman EA, Attarian R, Cho B, Biet F, Enns R, Bressler B. Immunogenicity of Mycobacterium avium subsp. paratuberculosis proteins in Crohn's disease patients. *Scand J Gastroenterol* 2011; **46**: 30-39 [PMID: 20735153 DOI: 10.3109/00 365521.2010.513061]

61   **Bradburn MJ**, Deeks JJ, Berlin JA, Russell Localio A. Much ado about nothing: a comparison of the performance of meta-analytical methods with rare events. *Stat Med* 2007; **26**: 53-77 [PMID: 16596572 DOI: 10.1002/sim.2528]

62   **Bhaumik DK**, Amatya A, Normand SL, Greenhouse J, Kaizar E, Neelon B, Gibbons RD. Meta-Analysis of Rare Binary Adverse Event Data. *J Am Stat Assoc* 2012; **107**: 555-567 [PMID: 23734068 DOI: 10.1080/01621459.2012.664484]

63   **Bai O**, Chen M, Wang X. Bayesian Estimation and Testing in Random Effects Meta-analysis of Rare Binary Adverse Events. *Stat Biopharm Res* 2016; **8**: 49-59 [PMID: 27127551 DOI: 10.1080/194 66315.2015.1096823]

64   **Ng SC**. Emerging Trends of Inflammatory Bowel Disease in Asia. *Gastroenterol Hepatol* (NY) 2016; **12**: 193-196 [PMID: 27231449]

65   **WHO**. Global tuberculosis report 2017. Accessed on March 27, 2018. Available from: URL: http://www.who.int/tb/publications/

global_report/en/

66   **Zwerling A**, Behr MA, Verma A, Brewer TF, Menzies D, Pai M. The BCG World Atlas: a database of global BCG vaccination policies and practices. *PLoS Med* 2011; **8**: e1001012 [PMID: 21445325 DOI: 10.1371/journal.pmed.1001012]

67   **Sartor RB**. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat Clin Pract Gastroenterol Hepatol* 2006; **3**: 390-407 [PMID: 16819502 DOI: 10.1038/ncpgasthep0528]

Liposarcoma of the stomach: Report of two cases and review of the literature

Wen-Zhe Kang, Li-Yan Xue, Gui-Qi Wang, Fu-Hai Ma, Xiao-Long Feng, Lei Guo, Yang Li, Wei-Kun Li, Yan-Tao Tian

Wen-Zhe Kang, Fu-Hai Ma, Yang Li, Wei-Kun Li, Yan-Tao Tian, Department of Pancreatic and Gastric Surgery, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China

Li-Yan Xue, Xiao-Long Feng, Lei Guo, Department of Pathology, National Cancer Center/ National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China

Gui-Qi Wang, Department of Endoscope, National Cancer Center/ National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China

ORCID number: Wen-Zhe Kang (0000-0001-9965-8109); Li-Yan Xue (0000-0001-5185-0126); Gui-Qi Wang (0000-0001-7767-1564); Fu-Hai Ma (0000-0003-2437-6881); Xiao-Long Feng (0000-0003-4475-9513); Lei Guo (0000-0002-3110-8167); Yang Li (0000-0002-4549-7087); Wei-Kun Li (0000-0002-3883-1497); Yan-Tao Tian (0000-0001-6479-7547).

Author contributions: Tian YT, Xue LY, and Wang GQ designed the report; Ma FH, Guo L, Li Y, and Li WK collected the patient's clinical data; and Kang WZ and Feng XL analyzed the data and wrote the paper.

Abstract

Liposarcoma of the stomach is extremely rare, and only 37 cases have been reported worldwide. We herein report two cases of liposarcoma of the stomach. The first patient was referred to our hospital with upper abdominal discomfort. The endoscopic examination revealed a tumor mass about 3 cm in diameter. The patient underwent a partial gastrectomy and had an uneventful recovery. The histopathological examination revealed a well-differentiated liposarcoma. The second patient had symptoms of upper abdominal discomfort combined with nausea and anorexia. Several palpable masses were found with endoscopy. Endoscopic submucosal dissection

was the treatment used, and the postoperative course was uneventful. The histopathological diagnosis was a well-differentiated liposarcoma. The two patients did not undergo any adjuvant therapy. They are both currently in good condition without recurrence. Therefore, we believe that the outcome of liposarcoma of the stomach is positive, and surgical resection may be the first choice for treatment at present.

Key words: Pathology; Signs and symptoms; Diagnosis; Liposarcoma; Therapeutics

Core tip: Liposarcoma of the stomach is extremely rare, and only 37 cases have been reported in the literature. We herein report two cases and review the literature. These cases might contribute to improving our understanding of the etiology, diagnosis, treatment strategies, and outcome of liposarcoma of the stomach. This report can also serve as a reminder to gastroenterologists, surgeons, and pathologists who encounter liposarcoma of the stomach in their clinical practice.

INTRODUCTION

Liposarcoma is one of the most common mesenchymal neoplasms[1], and liposarcomas are classified histologically into five subtypes[2]. However liposarcoma of the stomach is rare, and only 37 cases have been reported in the literature. Liposarcoma of the stomach is mainly located in the antrum, and it is usually of submucosal origin. Definitive diagnosis is reached only by histopathological examination. Because of the low incidence of this tumor, treatment of gastric liposarcoma is still not well-standardized. However, the prognosis may remain satisfactory if the condition is diagnosed early and treated appropriately. Herein, two cases of liposarcoma of the stomach are described, and we also discuss the histopathological types, etiology, diagnosis, and treatment strategies in this report.

CASE REPORT

In the Chinese Academy of Medical Sciences Cancer Hospital we encountered two cases, one in 2009 and one in 2016.

Case 1
The first patient was a 45-year-old woman who presented with the symptom of upper abdominal discomfort, which she had experienced for 6 mo. She complained of abdominal pain without any fever or gastrointestinal bleeding. During the physical examination, no special physical signs were found.

The gastroscopy revealed a large tumor mass about 5 cm in diameter located in the junction of the body and fundus of the stomach; it had been considered a benign tumor. Computed tomography confirmed a spherical tumor in the stomach, which was approximately 5.6 cm × 4.2 cm × 3.5 cm in size. The border of the tumor was clear and presented a significantly strengthened edge, and the center of the tumor was inhomogeneous. There were no visible signs of metastatic disease. Upper gastrointestinal imaging also found a circular tumor with smooth edges. The patient had no distinctive past medical history and denied any relevant family history. On March 30, 2009, the patient underwent a resection of the stomach tumor, and surgeons resected part of the omentum. An intraoperative pathology freezing study revealed mesenchymal neoplasms.

She had an uneventful recovery and was discharged after 9 d. The patient did not undergo any adjuvant treatment. She has remained under close follow-up supervision and is currently disease free.

The histopathological examination revealed a well-differentiated liposarcoma measuring 6 cm × 5 cm × 4 cm, which had infiltrated the muscle and serosal layers of the gastric wall (Figure 1). The immunohistochemistry finds were S-100+, CD34++, SMA+, Desmin++, CD117-, HMB45-, and Ki-67 < 1%. Fluorescent *in situ* hybridization (FISH) detection showed amplification of the MDM2 gene (Figure 2).

Case 2
A 69-year-old man was admitted to our department because of upper abdominal discomfort combined with nausea and anorexia that he had been experiencing for about 6 mo. During this period he lost 10 kg in weight. At first he pursued treatment with traditional Chinese medicine, and his symptoms were relieved. He underwent pituitary surgery in 2014 because of a pituitary tumor, and he had suffered from hypertension for 30 years. As a result of regular medication, his blood pressure was well controlled. His family history was unremarkable.

Our hospital's endoscopic examination showed that a limited knurl was distributed from the lower part of the gastric body to the corner of the stomach (Figure 3A), and a knurl was also found in the gastric fundus (figure 3B). Multiple biopsies were obtained, but they were all superficial and showed only unspecific inflammation of the gastric mucosa. Gastric endoscopic ultrasound (EUS) examination revealed that the tumor was mainly located in the submucosa of the gastric wall and was potentially a liposarcoma (Figure 4). Computed tomography confirmed a fat density tumor about 5.1 cm × 2.8 cm in size. No hepatic metastasis or nodal involvements were detected.

Figure 1 Histological finding. Low magnification shows that the immature fat cells are interspersed among smooth muscle tissues (A: HE, × 40 and B: HE; × 100). The large nuclear dark-stained lipoblast, which appears as a mononuclear or multinucleated cell with one or more cytoplasmic vacuoles, are seen under high magnification (C; HE; × 200 and D: HE; × 400).

Figure 2 Fluorescence *in situ* hybridization detection shows amplification of *MDM2* gene (A and B), case 1.

On August 1, 2016, an endoscopic submucosal dissection (ESD) was performed (Figure 5). During the operation, we found that the surface of the tumor was complete and smooth, and the substrate was sturdy. The operation was successful without any complications. The postoperative course was uneventful, and the patient was discharged on postoperative day 7. He did not undergo any adjuvant treatment and remained free of metastasis 20 mo after surgery.

The histopathological diagnosis was a well-differentiated liposarcoma (Figures 6 and 7). FISH testing demonstrated amplification of the MDM2 gene (Figure 8).

DISCUSSION

Liposarcoma, a kind of malignant tumor of mesenchymal origin, is one of the most common soft tissue sarcomas[1]. However, liposarcoma of the stomach is extremely rare. The first case was reported by Abrama and Tuberville in 1941, and until now only 37 cases (with a mean age of 57.0 years) have been reported worldwide (Table 1).

According to the 2013 WHO classification of soft tissue tumors, liposarcoma is a malignant fat cell tumor that can be histologically subdivided into the following five types: atypical lipomatous tumor/well differentiated liposarcoma, dedifferentiated liposarcoma, myxoid liposarcoma, pleomorphic liposarcoma, and liposarcoma, not otherwise specified[2].

Both of our cases had well-differentiated liposarcomas. Well-differentiated liposarcoma (including atypical lipomas) is the most common subtype, accounting for about 40%-45% of all liposarcomas[3]. Under the

Figure 3 A limited knurl was distributed from lower part of the gastric body to the corner of the stomach (A), and a knurl was also found in the gastric fundus (B).

Figure 4 Endoscopic ultrasound examination located the tumor mainly in the submucosa of the gastric wall.

Figure 5 Process of the endoscopic submucosal dissection.

microscope, the tumor cells look like normal fat cells. This kind of liposarcoma is usually a low-grade tumor in the early stages and grows slowly[4]. There is a risk of local recurrence but a low potential for metastasis[5]. Dedifferentiated liposarcomas and well-differentiated liposarcomas are related[1]. Approximately 10% of dedifferentiated liposarcomas are recurrences of well-differentiated liposarcomas[3]. The second most common subtype is myxoid liposarcoma, which represents about a third of all liposarcomas[3]. Myxoid liposarcoma is characterized by a myxoid matrix[6]. Under the microscope, its cells look less normal and may have a high grade component. Tumor cells infiltrate blood vessels in the fibromyxoid stroma that form characteristic clusters or branches. Therefore, we usually categorize myxoid liposarcomas as intermediate to high

Table 1 Review of literature

	Year	Author	Age	Sex	Treatment	Size (cm)	Histologic subtype	Outcome
1	1941	Abrams et al[23]	52	M	Exploratory laparotomy	Entire length of the stomach minor curvature	Unknown	DOD in 4 mo
2	1955	Hohf et al[20]	77	M	S + radiation	15 × 8 × 6 antrum	Unknown	WR in 8 mo
3	1965	Hawkins et al[21]	86	M	S	10 × 10 fundus	MY	WR in 24 mo
4	1968	Orita et al[22]	42	M	S	1.2 × 1.0 × 1.0 body	Unknown	WR 60 mo
5	1969	Souhei Suzuki et al[23]	42	F	S	13 × 11 × 9.5	Mixed	WR
6	1983	Hiroaki et al[24]	41	M	S	4.0 × 3.5 × 1.5	MY	DOD in 18 mo
7	1984	Lopez et al[28]	24	M	S	10 in diameter	MY	WR
8	1986	Kiyoshi Kagawa et al[23]	64	F	Tumor resection	About hen-egg	WD	WR
9	1986	Shokouh-Amiri et al[29]	15	M	S	30 × 20 Greater curvature	MY	WR 8 mo
10	1986	Laky et al[9]	67	F	P	5 × 2 × 1.5 antrum	Mixed	WR 12 mo
11	1988	Toshiki Hirose et al[23]	66	M	T	10 × 8 × 3	My	Dissemination
12	1990	Sacchiko Matsusaki[23]	42	F	S	about 600 g	MY	WR
13	1992	Costa et al[30]	70	F	S	9 in diameter	WD	WR
14	1993	Ryou Mochituki et al[23]	56	F	P	Child's head 1300 g	WD	Unknown
15	1993	Yoshiaki Suzuki et al[23]	59	M	T	/	Dedifferentiated	Unknown
16	1993	Ferrozzi et al[25]	58	M	Tumor resection	25 × 20 × 8 antrum	Pleomorphic	Unknown
17	1995	Shigeharu Suziki et al[23]	57	M	S + chemotherapy	4.8 in diameter	WD	WR
18	1995	Yamamoto et al[26]	58	M	P + endoscopic resection	1.3 × 0.5 Greater curvature	Mixed	WR 12 mo
19	1996	Mitsuyoshi Sakayani[23]	72	F	T	17.5 × 7.5 × 1.3; 1700 g	Pleomorphic	Unknown
20	1997	Tsutomu Andou et al[23]	68	F	T	10.6 × 4	WD	Unknown
21	1997	Masahiro Matsuzawa[23]	34	F	Distal gastrectomy	3.5 × 3 × 3	MY	Unknown
22	1997	Lopez-Negrete[15]	74	F	T	15 in diameter minor curvature	Mixed	Sudden death
23	1998	Seki et al[11]	68	F	T	10.5 × 5.5 × 4 body	WD	WR 13 mo
24	2000	Philipps et al[27]	74	F	S	3.4 × 1.3 × 0.5 antrum	MY	WR 15 d
25	2002	Hisanobu Saegusa et al[23]	34	F	S	4 × 2.8 minor curvature	WD	WR in 36 mo
26	2005	Noushin et al[15]	62	M	S	7 × 6 minor curvature	WD	Unknown
27	2007	Konstantinos et al[6]	68	M	T	9 × 4 fundus	WD	WR in 24 mo
28	2007	Michiels et al[7]	27	F	Subtotal gastrectomy, liver, diaphragm, pancreas, spleen, pericardium; adjuvant chemotherapy	30 × 20 (5 kg) minor curvature	Pleomorphic	DOD in 16 mo
29	2012	Mohamed et al[11]	51	M	T	9 × 7.5 × 5 antrum	WD	WR in 12 mo
30	2013	Akin et al[1]	59	F	Distal gastrectomy	4 × 3 × 2.5 antrum	WD	WR in 12 mo
31	2014	Kim et al[18]	46	F	Laparoscopic, distal gastrectomy; adjuvant treatment	7 in diameter body	WD	Unknown
32	2015	Abderrahman et al[3]	70	M	Antrectomy + adjuvant therapy	36 in diameter antrum	MY	DOD in 11 mo
33	2016	Matone et al[4]	76	M	Laparoscopic +P	7.5-7.0 in diameter antrum	WD	WR in 6 mo
34	2017	Jiang et al[14]	55	F	P + tail of pancreas and spleen was resected	1.5 in diameter fundus	WD	WR in 48 mo
35	2017	Hisata et al[13]	79	F	Surgery for the cardiac tumor	0.5-1.0 in diameter greater curvature	Dedifferentiated	DOD in 55 d
36	2017	Tomofuji et al[31]	61	F	Laparoscopic total gastrectomy	5 in diameter fundus	WD	WR in 14 mo
37	2018	Girardot-Miglierina et al[32]	/	/	/	Gastro-esophageal junction	Unknown	Unknown
38	2016	Our case	70	M	Endoscopic resection	6 × 3.5 × 2 minor curvature	WD	WR in 20 mo
39	2009	Our case	45	F	Tumor resection	6 × 5 × 4 body	WD	WR in 9 yr

DOD: Death of disease; WR: Without recurrence; MY: Myxoid liposarcoma; Mixed: Mixed type liposarcoma; WD: Well-differentiated liposarcoma; S: Subtotal gastrectomy; P: Partial gastrectomy; T: Total gastrectomy.

grade tumors. Pleomorphic liposarcoma is considered the least common subtype and has been properly characterized only recently. It accounts for approximately 5% of liposarcomas and is a highly malignant lesion[7]. Pleomorphic liposarcoma is characterized by increased mitotic activity and hemorrhage as well as necrosis[8].

Figure 6 Gross specimen of the tumor.

Microscopically, the tumor cells consist of varying amounts of pleomorphic lipoblasts. Pleomorphic sarcoma consists of highly shaped spindle cells, round cells, polygonal cells, or giant tumor cells. Several grading systems have been developed to classify the tumors and to differentiate between low-grade and high-grade tumors.

These tumors can appear anywhere in the body but often occur in the limbs and retroperitoneal space, and the rest occur in the head and neck, abdominal wall, chest wall, and other areas. Liposarcomas hardly ever occurs in organs[6] and are mainly found in adults, with a peak incidence between the age of 50 and 65 years[4]. The origin of gastric liposarcomas is likely the proliferation of undifferentiated mesenchymal cells within the submucosa and the tunica muscularis layer of the stomach[9]. Gastric liposarcomas are characterized by an exophytic growth that adheres to the gastric wall, and the typical location of gastric liposarcomas is the antrum. According our statistics, approximately 30.8% (8/26) of gastric liposarcomas are located in the antrum, and they are usually of submucosal origin. In addition, 23.1% (6/26) of gastric liposarcomas are located in the minor curvature, 15.4% (4/26) in the fundus, 15.4% (4/26) in the body, 11.5% (3/26) in the greater curvature, and 3.8% (1/26) in the gastro-esophageal junction. The diameter of the tumors described in the literature varies from 0.5 to 36 cm.

The etiology of liposarcoma is not yet clear; environmental factors, radiation, genetic variation, and immune defects are potential risk factors[10]. Some patients have a familial history of soft tissue tumors[4], which suggests genetic factors may play an important role in the occurrence of gastric liposarcoma.

Because the tumor develops within the gastric wall, it presents an extra-luminal growth, and the patient can remain asymptomatic for a long time. The symptoms of gastric liposarcoma range from dyspepsia, nausea, vomiting, anorexia, abnormal bowel movements, asthenia, and epigastric abdominal pain to upper gastrointestinal tract bleeding. The type of symptom that appears depends on the location and size of the tumor and the presence of ulcerations[3]. Space-occupying lesions of the stomach or abdominal cavity contribute to the appearance of clinical symptoms[1]. When the submucosal mass extrudes into the lumen, it can cause traumatic and inflammatory changes and result in necrosis, ulceration, and hemorrhage[11]. For patients with giant tumors, the main clinical sign may be the presence of a large abdominal mass of unknown origin[3]. In our cases, the main clinical sign in both patients was epigastric abdominal pain that continued for longer than 6 mo. In both cases, the typical exophytic growth explains the lack of specific gastrointestinal symptoms and the delayed diagnosis[12]. Some cases of gastric liposarcoma can involve other organs synchronously, and unique symptoms may be present[13,14].

Unfortunately, because of the lack of specific symptoms, it is difficult to achieve an early diagnosis[7]. The diagnosis of gastric liposarcoma mainly relies on pathological examination. Cytogenetics and molecular biology provide effective tools for differentiating among types of lipomatous tumors[11]. Macroscopically, liposarcoma present intricate myxomatous zones, which include round cells, pleomorphous clearly differentiated lipoblastic aspects, and hemorrhagic areas[15]. Because endoscopic biopsies do not penetrate the submucosa, the diagnostic value of the endoscopy is unclear, and it is difficult to make a precise judgment on the basis of biopsy findings. Endoscopic biopsies may be useful when the tumor presents endoluminal development. With the guidance of EUS or abdominal ultrasound, biopsy may be possible, and a histological examination, immunohistochemistry, and a cytogenetic study can be performed[3]. Detection of MDM2 is probably important in diagnosis. In terms of imaging, computed tomography is considered the most informative examination. The presence of fat density areas is pathognomonic for fatty tumors, and an association with enhanced areas is highly suggestive of the diagnosis[16]. CT scans can also show secondary lesions in the liver, lung, peritoneum, or other places.

Currently, the main therapy for gastric liposarcoma is surgical removal. The type of gastrectomy chosen depends on the location of the tumor. According to the rules of sarcoma resection, surgeons should resect the tumor with a wide margin of healthy tissue around it and make sure there is no remaining tumor tissue[3]. Lymph node dissection may be unnecessary[17]. One of our patients underwent a ESD. Due to the lack of sufficient data, the advantages and disadvantages of this method are unknown. In consideration of the successful application of ESD in early gastric cancer, we believe this method is available for a low-grade tumor in the early stages. Chemotherapy and radiotherapy combined with surgery have been successful in most malignant tumors; however, we still cannot develop a guideline for chemotherapy and radiotherapy in patients with gastric liposarcoma. There is very little information in the literature about the use of chemotherapy for gastric liposarcoma. Because of a high local recurrence rate

Figure 7 Histological findings. Under low magnification, irregular cell cluster nests were seen around the mature adipocytes (A: HE; × 20 and B: HE; × 40); intermediate magnification and high magnification showed that the heteromorphic cell nests consisted of large nuclear dark-stained tumor cells, with distinct cell shapes, irregular cell morphologies, and visible Mitosis icon (C: HE; × 100, D: HE; × 100, E: HE; × 200, and F: HE; × 400).

Figure 8 Fluorescence in situ hybridization detection shows *MDM2* gene amplification, case 2.

of 70%-90% for high-grade soft-tissue sarcomas[18], adjuvant therapy may be necessary. On the contrary, Matone *et al*[4] hold the opinion that there is currently no evidence that chemotherapy or radiotherapy improves survival rates. Three drugs, ifosfamide, doxo/epirubicin, and dacarbazine are active in the therapy of adult soft tissue sarcoma[7]; they provide a potential therapeutic pathway for gastric liposarcoma. Radiation therapy may be beneficial by killing tumor cells and reducing the chance of the tumor returning in the same location[3] and may be widely used in the treatment of sarcoma. Only six cases reported in the literature received adjuvant treatment. In our cases, patients did not undergo any adjuvant or neoadjuvant therapy. Both patients are free from recurrence after sarcoma resection.

The main prognostic factor for the primary tumor is histological type, and other factors include the scope and location of the tumor[7]. Kim *et al*[19] believe the main prognostic factor for the primary tumor is anatomical location. According to our statistics, mortality associated with gastric liposarcoma is usually found in cases of dedifferentiated liposarcoma, myxoid liposarcoma, pleomorphic liposarcoma, and mixed type liposarcoma. Of the 37 cases described, six patients died of the disease, while the outcome for nine patients is not known. Their survival time ranged from sudden death to 18 mo (specifically, the times were immediate, 55 d, 4 mo, 11 mo, 16 mo, and 18 mo). Some studies reported that 30% of well-differentiated liposarcomas present with local recurrence; however, metastasis is hardly ever seen[1,4]. Pleomorphic liposarcoma is considered a highly malignant lesion and may indicate a poor outcome[7]. Due to the lack of sufficient data, we still cannot clearly determine the relationship between histological type and disease prognosis. The outcome of gastric liposarcoma is still unclear, and further study is needed.

From the reported cases and literature review[20-32], we conclude that liposarcoma is rarely seen in the viscera, especially the stomach. Diagnosis of this tumor mainly depends on histopathological examination. Gastric liposarcomas are extremely rare tumors for which there is no therapeutic consensus. Although medications and devices have improved in recent years, surgery may be the most reasonable treatment, and the role of adjuvant treatment is not clearly defined. The prognosis is still unclear, and more research is needed. However, we

believe that if the tumor is diagnosed early and treated effectively, the postoperative outcome may be positive.

ARTICLE HIGHLIGHTS

Case characteristics
Epigastric abdominal pain that continued for longer than 6 mo.

Clinical diagnosis
Gastrointestinal stromal tumor (GIST) and gastric lipoma.

Differential diagnosis
Differential diagnosis: GIST and gastric lipoma. Definitive diagnosis is reached only by histopathological examination.

Laboratory diagnosis
Gastric liposarcoma.

Imaging diagnosis
Computed tomography: Gastric lipoma.

Pathological diagnosis
Gastric liposarcoma.

Treatment
Partial gastrectomy and endoscopic submucosal dissection.

Related reports
The first case was reported by Abrama and Tuberville in 1941, and until now only 37 cases have been reported worldwide (Table 1).

Term explanation
Two cases of gastric liposarcoma are reported and a review of the literature.

Experiences and lessons
Diagnosis of gastric liposarcoma mainly depends on histopathological examination, and surgery may be the most reasonable treatment.

REFERENCES

1   **Bostanoğlu A**, Yıldız B, Kulaçoğlu S, Avşar F. Primary liposarcoma of the stomach. *Turk J Gastroenterol* 2013; **24**: 167-169 [PMID: 23934465 DOI: 10.4318/tjg.2013.0510]
2   **Fletcher CDM**, Bridge JA, Hogendoom PCW. World Health Organization classification of tumous. Pathology and genetics of tumours of soft tissue and bone. Lyon: IARC Press, 2013: 10-238
3   **Elhjouji A**, Jaiteh L, Mahfoud T, Belhamidi S, Bounaim A, AitAli A, Sair K, Zentar A. Giant Gastric Liposarcoma: A Fatal Exceptional Location. *J Gastrointest Cancer* 2016; **47**: 482-485 [PMID: 26558372 DOI: 10.1007/s12029-015-9779-z]
4   **Matone J**, Okazaki S, Maccapani GN, Amancio TT, Filippi RZ, Macedo AL. Giant gastric lipossarcoma: case report and review of the literature. *Einstein (Sao Paulo)* 2016; **14**: 557-560 [PMID: 28076606 DOI: 10.1590/S1679-45082016RC3770]
5   **Wu JM**, Montgomery E. Classification and pathology. *Surg Clin North Am* 2008; **88**: 483-520, v-vi [PMID: 18514695 DOI: 10.1016/j.suc.2008.03.007]
6   **Tepetes K**, Christodoulidis G, Spyridakis ME, Nakou M, Koukoulis G, Hatzitheofilou K. Liposarcoma of the stomach: a rare case report. *World J Gastroenterol* 2007; **13**: 4154-4155 [PMID: 17696242 DOI: 10.3748/wjg.v13.i30.4154]
7   **Michiels A**, Hubens G, Ruppert M, Balliu L, Vaneerdeweg W. Giant liposarcoma of the stomach involving the mediastinum. *Acta Chir Belg* 2007; **107**: 468-471 [PMID: 17966553 DOI: 10.1080/00

015458.2007.11680102]
8   **Einarsdóttir H**, Skoog L, Söderlund V, Bauer HC. Accuracy of cytology for diagnosis of lipomatous tumors: comparison with magnetic resonance and computed tomography findings in 175 cases. *Acta Radiol* 2004; **45**: 840-846 [PMID: 15690614 DOI: 10.1080/02841850410008180]
9   **Laky D**, Stoica T. Gastric liposarcoma. A case report. *Pathol Res Pract* 1986; **181**: 112-117 [PMID: 3703739 DOI: 10.1016/S0344-0338(86)80202-3]
10   **Arndt CA**, Crist WM. Common musculoskeletal tumors of childhood and adolescence. *N Engl J Med* 1999; **341**: 342-352 [PMID: 10423470 DOI: 10.1056/NEJM199907293410507]
11   **Hamdane MM**, Brahim EB, Salah MB, Haouas N, Bouhafa A, Chedly-Debbiche A. Giant gastric lipoma mimicking well-differentiated liposarcoma. *Gastroenterol Hepatol Bed Bench* 2012; **5**: 60-63 [PMID: 24834200]
12   **Seki K**, Hasegawa T, Konegawa R, Hizawa K, Sano T. Primary liposarcoma of the stomach: a case report and a review of the literature. *Jpn J Clin Oncol* 1998; **28**: 284-288 [PMID: 9657017]
13   **Hisata Y**, Tasaki Y, Kozaki S, Yamada T. A case of dedifferentiated liposarcoma of the heart and stomach. *Int J Surg Case Rep* 2017; **41**: 36-38 [PMID: 29031176 DOI: 10.1016/j.ijscr.2017.10.001]
14   **Jiang HF**, Ying XJ. Synchronous liposarcoma of pancreas and stomach: a case report and literature review. Int Surg J 2017, 4: 780-783 [DOI: 10.18203/2349-2902.isj20170231]
15   **Noushin AM**, Safaei M. Primary Liposarcoma of the Stomach: A Rare Mesenchymal Tumor. Medical J Islamic Republic of lran 2005, 19: 275-278
16   **López-Negrete L**, Luyando L, Sala J, López C, Menéndez de Llano R, Gomez JL. Liposarcoma of the stomach. *Abdom Imaging* 1997; **22**: 373-375 [PMID: 9157853 DOI: 10.1007/s002619900213]
17   **Mutter D**, Marescaux J. Gastrectomies pour lesions benignes. Techniques chirurgicales-Appareil digestif 2001, 16: 40-320 [DOI: 10.1016/S1282-9129(01)72065-X]
18   **Bramwell VH**. Adjuvant chemotherapy for adult soft tissue sarcoma: Is there a standard of care? *J Clin Oncol* 2001; **19**: 1235-1237 [PMID: 11230463 DOI: 10.1200/JCO.2001.19.5.1235]
19   **Kim MJ**, Gu MJ, Choi JH, Kim SW, Kim KO. Gastric liposarcoma presenting as a huge pedunculated polyp. *Endoscopy* 2014; **46** Suppl 1 UCTN: E441-E442 [PMID: 25314184 DOI: 10.1055/s-0034-1377501]
20   **Hohf RP**, Engel KL, Capos NJ. Liposarcoma of the stomach. *Ann Surg* 1955; **142**: 1029-1033 [PMID: 13269065]
21   **Hawkins PE**, Terrell GK. Liposarcoma of the stomach: a case report. *JAMA* 1965; **191**: 758-759 [PMID: 14245524 DOI: 10.1001/jama.1965.03080090072023]
22   **Orita K**, Kokumai Y, Kawada K, Takagi S, Kawahara T. Liposarcoma of stomach: report of a case. *Acta Med Okayama* 1968; **22**: 167-173 [PMID: 4239074]
23   **Hisanobu S**, Toshiki S, Yasuhide O, Tetsuya I, Kouichi M, Ryugo O, Kenji S, Taiji A. Primary Gastric Liposarcoma, Report of a Case and Review of the Literature. *Gastroenterol Endosc* 2002; **44**: 1168-1174 [DOI: 10.11280/gee1973b.44.1168]
24   **Seki H**, Kataba Y, Okamura R, Namatame K, M Hagiwara M. Metastatic Liposarcoma of the Stomach Report of a Case. *Gastroenterological Endoscopy* 1983; **4**: 628-635 [DOI: 10.11280/gee1973b.25.628]
25   **Ferrozzi F**, Bova D, Garlaschi G. Gastric liposarcoma: CT appearance. *Abdom Imaging* 1993; **18**: 232-233 [PMID: 8508081 DOI: 10.1007/BF00198110]
26   **Yamamoto K**, Teramae N, Uehira H, Wakabayashi N, Fukuda S, Kodama T, Kashina K, Tsuchihashi Y. Primary liposarcoma of the stomach resected endoscopically. *Endoscopy* 1995; **27**: 711 [PMID: 8903993 DOI: 10.1055/s-2007-1005798]
27   **Philipps B**, Lörken M, Manegold E, Kasperk R, Schumpelick V. [Primary liposarcoma of the stomach wall--a rare mesenchymal tumor]. *Chirurg* 2000; **71**: 334-336 [PMID: 10789053]
28   **López de la Riva M**, Urquijo Tomatti H. [Gastric liposarcoma. Presentation of a case]. *Rev Esp Enferm Apar Dig* 1984; **66**:

335-338 [PMID: 6515090]

29 **Shokouh-Amiri MH**, Hansen CP, Moesgaard F. Liposarcoma of the stomach. A case report. *Acta Chir Scand* 1986; **152**: 389-391 [PMID: 3739551]

30 **Costa e Silva N**, Melo CM, Naves EB, Dias MA. [Gastric liposarcoma: report of a case]. *Rev Hosp Clin Fac Med Sao Paulo* 1992; **47**: 89-91 [PMID: 1340019]

31 **Tomofuji K**, Watanabe J, Ishida N, Kajiwara S. Gastric liposarcoma resected by laparoscopic total gastrectomy to achieve a wide surgical margin. *BMJ Case Rep* 2017; **2017** [PMID: 29212871 DOI: 10.1136/bcr-2017-221703]

32 **Girardot-Miglierina A**, Clerc D, Suter M. Gastric liposarcoma in a patient with severe obesity. *Ann R Coll Surg Engl* 2018; **100**: e88-e90 [PMID: 29484946 DOI: 10.1308/rcsann.2018.0018]

World Journal of

Gastroenterology

World Journal of Gastroenterology

World Journal of *Gastroenterology*

Contents

Contents

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Yasemin H Balaban, MD, Doctor, Professor, Department of Gastroenterology, Hacettepe University, Ankara 06100, Turkey

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol, WJG,* print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 642 experts in gastroenterology and hepatology from 59 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

Liver transplantation and alcoholic liver disease: History, controversies, and considerations

Claudio Augusto Marroni, Alfeu de Medeiros Fleck Jr, Sabrina Alves Fernandes, Lucas Homercher Galant, Marcos Mucenic, Mario Henrique de Mattos Meine, Guilherme Mariante-Neto, Ajacio Bandeira de Mello Brandão

Claudio Augusto Marroni, Sabrina Alves Fernandes, Lucas Homercher Galant, Guilherme Mariante-Neto, Ajacio Bandeira de Mello Brandão, Graduate Program in Medicine: Hepatology, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre 90430-080, RS, Brazil

Claudio Augusto Marroni, Alfeu de Medeiros Fleck Junior, Sabrina Alves Fernandes, Lucas Homercher Galant, Marcos Mucenic, Mario Henrique de Mattos Meine, Guilherme Mariante-Neto, Ajacio Bandeira de Mello Brandão, Liver Transplant Adult Group, Irmandade da Santa Casa de Misericórdia de Porto Alegre, Porto Alegre 90035-072, RS, Brazil

Sabrina Alves Fernandes, Nutrition at the Centro Universitário Metodista (IPA), Porto Alegre 90420-060, RS, Brazil

ORCID number: Claudio Augusto Marroni (0000-0002-1718-6548); Alfeu de Medeiros Fleck Jr (0000-0002-0424-6919); Sabrina Alves Fernandes (0000-0001-8504-603X); Lucas Homercher Galant (0000-0002-4333-1403); Marcos Mucenic (0000-0001-9389-2236); Mario Henrique de Mattos Meine (0000-0001-9914-6381); Guilherme Mariante-Neto (0000-0002-1580-3725); Ajacio Bandeira de Mello Brandão (0000-0001-8411-5654).

Author contributions: All authors reviewed and analyzed the literature and contributed to the writing of the manuscript; Marroni CA also provided the overarching intellectual input into the study design and edited the final version of the manuscript.

Conflict-of-interest statement: No potential conflicts of interest. No financial support.

Abstract

Alcohol consumption accounts for 3.8% of annual global mortality worldwide, and the majority of these deaths are due to alcoholic liver disease (ALD), mainly alcoholic cirrhosis. ALD is one of the most common indications for liver transplantation (LT). However, it remains a complicated topic on both medical and ethical grounds, as it is seen by many as a "self-inflicted disease". One of the strongest ethical arguments against LT for ALD is the probability of relapse. However, ALD remains a common indication for LT worldwide. For a patient to be placed on an LT waiting list, 6 mo of abstinence must have been achieved for most LT centers. However, this "6-mo rule" is an arbitrary threshold and has never been shown to affect survival, sobriety, or other outcomes. Recent studies have shown similar survival rates among individuals who undergo LT for ALD and those who undergo LT for other chronic causes of end-stage liver disease. There are specific factors that should be addressed when evaluating LT patients with ALD because these patients commonly have a high prevalence of multisystem alcohol-related changes. Risk factors for relapse include the presence of anxiety or depressive disorders, short pre-LT duration of

sobriety, and lack of social support. Identification of risk factors and strengthening of the social support system may decrease relapse among these patients. Family counseling for LT candidates is highly encouraged to prevent alcohol consumption relapse. Relapse has been associated with unique histopathological changes, graft damage, graft loss, and even decreased survival in some studies. Research has demonstrated the importance of a multidisciplinary evaluation of LT candidates. Complete abstinence should be attempted to overcome addiction issues and to allow spontaneous liver recovery. Abstinence is the cornerstone of ALD therapy. Psychotherapies, including 12-step facilitation therapy, cognitive-behavioral therapy, and motivational enhancement therapy, help support abstinence. Nutritional therapy helps to reverse muscle wasting, weight loss, vitamin deficiencies, and trace element deficiencies associated with ALD. For muscular recovery, supervised physical activity has been shown to lead to a gain in muscle mass and improvement of functional activity. Early LT for acute alcoholic hepatitis has been the subject of recent clinical studies, with encouraging results in highly selected patients. The survival rates after LT for ALD are comparable to those of patients who underwent LT for other indications. Patients that undergo LT for ALD and survive over 5 years have a higher risk of cardiorespiratory disease, cerebrovascular events, and *de novo* malignancy.

Key words: Alcoholic liver disease; Alcoholic hepatitis; Alcoholic cirrhosis; Alcoholism; Liver transplantation; Alcoholic recurrence; Controversies; Alcoholic abstinence; Relapse; Selection criteria

Core tip: Alcohol consumption accounts for 3.8% of annual global mortality worldwide. Cirrhosis is a common complication of alcoholic liver disease (ALD) and when end-stage liver disease is reached, the only chance of survival is liver transplantation (LT). There are controversies and ethical dilemmas associated with LT for ALD. This study reviews the history and controversies and considers the development of, indications for, and outcomes of LT in ALD, including severe acute alcoholic hepatitis. Relapse, therapeutic options, and outcomes are emphasized.

INTRODUCTION

Liver transplantation (LT) is the treatment of choice for patients with liver failure in end-stage liver disease, and it is their only chance of survival. As patients on LT waiting lists outnumber the number of LTs performed, and due to the high waiting list mortality rate, prioritization of individuals who are most likely to die without LT is needed. Access based on who is most likely to benefit from organ donation requires a legal, fair and ethical basis for the allocation[1-3].

Alcohol consumption accounts for 3.8% of global mortality and 4.6% of disability-adjusted life-years (DALYs) lost due to premature death[4]. Among the various harmful effects of alcohol, alcoholic liver disease (ALD) induces a wide spectrum of liver abnormalities, including simple steatosis, alcoholic hepatitis (AH) or steatohepatitis, progressive fibrosis, and ultimately alcoholic cirrhosis (AC) and/or hepatocellular carcinoma (HCC)[5].

One of the common causes of chronic liver disease, for which LT is potentially lifesaving, is ALD. This has been highly controversial from the beginning. The ever-increasing shortage of organs has accentuated the low priority given to patients with ALD, which is considered a "self-inflicted" condition. However, by improving the long-term survival rates (thereby making them similar to those for LT patients with other indications) and recognizing that alcoholism is a primary disease, ALD has become one of the most common indications for LT in Europe and North America, a situation thought unfathomable 30 years ago. Unfortunately, there are still many issues with the use of LT for ALD.

LT for ALD used to be associated with many ethical dilemmas, but this has recently changed as alcoholism is now understood to be a chronic, recurrent neurological disease process with a clear biological basis. Alcoholism is no longer primarily viewed to be a result of moral weakness and self-destructive behavior, but an addiction and dependence that should be considered from other perspectives[5].

ALD progression is dependent on patient characteristics, as well as drinking patterns. Abstinence is fundamental to the treatment of all forms of ALD, and the alcohol consumption relapse rate is lower than that expected in alcoholics. The consequences of excessive drinking after LT range from asymptomatic biochemical and histological abnormalities to graft failure and death.

HISTORICAL PERSPECTIVE, CONTROVERSIES, AND CONSIDERATIONS

History

LT for ALD has been a controversial situation from the beginning because of the ever-increasing demand for donor organs and the inadequate rate of organ donation, combined with the concern that patients with alcoholism might relapse, thereby damaging the transplanted liver. There was an apprehension that the outcome of LT in these patients may not be the same as for LT patients with other indications.

Of the first ten patients who underwent LT, performed

by Starzl (before the advent of cyclosporine), nine did not survive the first 4 mo. This poor initial outcome was attributed to excessive alcohol consumption causing significant extrahepatic organ damage (such as pancreatitis, cardiomyopathy, cerebral dysfunction, and poor nutritional status). This was probably due to the selection of critically ill patients who were too sick to improve even with LT, and ALD was then considered a predictor of poor LT outcomes compared to other indications[6,7]. In 1984, Scharschmidt reported on the experience of four transplant centers that had performed 540 LTs in the United States and Western Europe. The 3-year survival rate for the 20 patients who underwent LT after 1980 was 20%; non-AC cirrhosis was associated with an impressive 42% survival rate[8].

In the United Kingdom, the University of Cambridge Department of Surgery at Addenbrooke's Hospital and the Liver Unit at King's College Hospital, London, started the Liver Transplant program in a joint collaborative endeavor in 1968[9], involving Roy Calne and Roger Williams. They have stated that patients with AC are seldom suitable for transplantation since they are often malnourished and particularly prone to precipitous clinical deterioration often provoked by infections, and there are often doubts as to their ability to control their drinking again after the transplant[9].

Between 1968 and 1987, 325 LTs were carried out, eight of which were for AC. Active alcoholism was a specific contraindication. All of them died before 25 wk, except for the last two, who died at 48 wk (around the time of publication of the report on the 325 patients); only one returned to drinking alcohol[10].

The United States National Institutes of Health (NIH) Consensus Conference on Liver Transplantation in 1983 concluded that ALD is an appropriate indication for LT, provided that the patient is judged likely to abstain from alcohol after LT[6]. Following this, there was an increase in the number of LTs being performed for ALD. However, the conference attendees still considered and predicted that ALD would be a marginal indication for LT.

The first positive data published on the survival rate of ALD patients after LT, in comparison to patients with other indications, were reported in 1988[11]. Starzl reported a 73% 1-year survival rate among 41 patients when cyclosporine was used as the main immunosuppressive drug[12], and only 3% of these patients had relapsed to alcoholism. This was a convincing argument in favor of LT for ALD.

In 1991, the US Health Care Financing Administration identified ALD as one of the seven conditions for which it approved payment for LT, but it recommended a "significant" period of abstinence before patients with alcoholism underwent the procedure, as well as the availability of a reasonable social support system. Beresford *et al*[12] proposed a selection method for identifying alcoholic patients who were suitable for LT. Furthermore, Lucey *et al*[13] reported on a multidisciplinary collaboration of transplant hepatologists, surgeons, and psychiatrists that identified psychosocial predictors of long-term sobriety and compliance after LT among patients with alcoholism.

The appropriateness of LT for ALD was confirmed in European and American centers in the early 90s, with 1-year survival rates being 66%-96%. There was increasing evidence that most ALD patients selected for LT have similar, if not better, survival rates compared to those who undergo LT for other indications (1-year survival rate of 86%)[14-18].The NIH workshop in 1996 on LT for ALD patients concluded that LT provides good outcomes for alcoholic patients and that relapse rates after LT were lower if the patients had successfully completed a conventional alcohol rehabilitation program prior to LT[7].

The European Liver Transplant Registry (ELTR)[19] accumulated information on LTs from 1968-2015 based on a progressive increase of cases. Among all the LT cases, the ELTR reported a rate of cases involving cirrhosis of 46%-55%, a rate of cases involving AC of 9%-44%, and a rate of cases involving AC + hepatitis C virus (HCV) infection of 2%-3%. In this period, viral cases increased from 8% to 27%, primary biliary cirrhosis cases decreased from 29% to 7%, and autoimmune hepatitis cases decreased from 10% to 5%[19]. There was a progressive increase in the number of indications for LT in the US after 2002, and the number of donors is much smaller than the number of patients who require LT.

Controversies

Patient selection for LT has always been a demanding responsibility for transplantation professionals. Less than 4% of AC patients were placed on an LT waiting list in the United States in 2007. In the United States, the majority of candidates with end-stage ALD who are eligible for referral for LT are not being referred. Kotlyar *et al*[20] analyzed data on alcohol abuse and dependence in the United States and found that the potential number of patients with ALD and decompensated AC who could be candidates for LT was 100000 patients/year. However, of these, only 10% (10000) were referred, and of these, 3673 were on the LT waiting list and 1200 underwent LT in 2018. Thus, every year, 4% of patients with decompensated AC were on the waiting list and 1.2% underwent LT. This pattern of referral may lead to as many as 12,000 deaths/year. There are multiple reasons for poor referral of these patients and these reasons occur at all levels[20].

LT for ALD still generates controversy because of both the perception that the patient's liver disease was self-inflicted and concerns related to relapse after LT. The general public, and even some practicing physicians outside the transplant community, view individuals with alcoholism as lower priorities for LT. Many specialists considered it unacceptable to "waste" grafts on individuals with alcoholism who were responsible for the harm caused to their liver, as they still consider alcoholism to be a bad habit, and there is a general reluctance to provide LT for these patients. Particularly in

the past, the naysayers believed that excessive alcohol consumption had multisystem organ consequences that precluded good surgery outcomes, that relapse-induced redevelopment of liver disease would occur, and that patients were unlikely to withstand the psychological issues caused by such a serious operation, resulting in poor compliance. However, neuroscience has shown that alcoholism is a chronic relapsing medical disease of the brain, and not a bad behavior. Ethical principles recommend active treatment of these patients, without discrimination[21-25].

LT provides the patients with a physiologically functioning liver and reverses the complications of end-stage liver disease, improving survival and quality of life, but it does not treat the underlying alcoholism and alcohol dependence, leading to the potential for relapse. The probability of long-term sobriety becomes robust only after 5 years of sustained abstinence[26-28].

Pre-LT alcohol abstinence represents one of the hottest and most controversial open questions on this topic. Many transplant centers use the criterion of 6 mo of abstinence to determine whether ALD patients should receive livers, known as the "6-mo rule". This rule has two purposes: to allow the patient's liver a chance to recover and to reduce the risk of alcohol consumption relapse. Six months is an arbitrary threshold and this period has never been shown to affect survival after LT, although there is a weak association between sobriety and LT outcome[29-32].

The rule was established in 1997, when the United Network for Organ Sharing (UNOS) had a meeting to discuss the criteria for placing adult ALD patients in need of a new liver on LT waiting lists. The recent guidelines of the American Association for the Study of Liver Diseases, European Association for the Study of the Liver, UNOS, and French Consensus Conference state that 6 mo of zero alcohol consumption before LT should no longer be an absolute rule or a defining factor to determine whether a patient is accepted as an LT candidate[29,33-36].

There is no firm consensus on the appropriate minimum duration of alcohol abstinence or on what constitutes good psychosocial criteria for placing patients on LT waiting lists. Physicians in the transplant community perceive selected patients with end-stage ALD as good candidates. An interval of sobriety prior to LT is very desirable from a medical point of view. Abstinence may markedly improve liver function[37].

ALD was the underlying etiology in the majority of patients who were removed from an LT waiting list following recompensation. There were only two independent predictors among ALD patients of recompensation/removal from the LT waiting list: A model for end-stage liver disease (MELD) score < 20 and serum albumin ≥ 32 g/L. The probability of recompensation was 70% when both factors were present when the patient was placed on the LT waiting list. Thus, it seems advisable for ALD patients to undergo a period of observation to check whether they have both favorable factors before

embarking on living donor LT[38].

Patients with ALD were placed on an LT waiting list based on the Ohio Solid Organ Transplantation Consortium (OSOTC) exception criteria. These criteria allow patients with low to medium risk of relapse to undergo LT after only 1-3 mo of abstinence. The results showed that the LT rate and short- and long-term post-LT survival are comparable between these patients and the general population of United States patients with AC who underwent LT[39].

Considerations

Compared to the traditional criteria for assessing risk of relapse, a careful selection process with more flexibility to evaluate eligibility on a case-by-case basis can lead to similar survival rates after LT. With regard to alcoholism, a mandatory period of abstinence is a poor predictor of relapse. Pre-LT abstinence does not reliably predict post-LT abstinence or compliance[37].

When considering whether to add patients to an LT waiting list, 3 mo of alcohol abstinence may be better than 6 mo. Patients with a lack of social support, active smoking, psychotic or personality disorders, or a pattern of nonadherence should be added to the waiting list only with reservation. Those who have a diagnosis of alcohol abuse, as opposed to alcohol dependence, may make better LT candidates. Patients who have regular addiction treatment appointments with a psychiatrist or psychologist also seem to do more favorably[20].

Alcohol addiction is considered a big problem after LT, while moderate alcohol consumption is underestimated by both patients and their healthcare providers. Sometimes there is even a recommendation to carefully assess the alcohol consumption of patients on LT waiting lists who have non-AC cirrhosis[40,41].

Alcohol consumption can complicate a patient's health after LT, and moderate consumption is important because it can aggravate metabolic syndrome and non-alcoholic fatty liver disease, which often develop, irrespective of alcohol consumption, as the side effect of post-LT immunosuppressive agents. This immunosuppressive treatment can lead to a risk of de novo malignancy that is 2-7-fold higher than usual after adjusting for age and gender, and 5- and 10-year incidence rates are estimated to be 10%-14.6% and 20%-32%, respectively[42,43]. Moderate alcohol consumption can have a negative effect on LT as it increases the risk of liver fibrosis, mainly in women, even if alcohol consumption is < 12 g/d[40-43].

Medical law experts are repeatedly reminded, albeit from a different point of view than those associated with medical practice, that US constitutional law prohibits discrimination against subgroups and differentiating individuals as being either worthy or unworthy of life. The exclusion of non-sober ALD patients from LT waiting lists discriminates against them and violates this United States constitutional law[44].

The fact that patients with acute liver failure after ecstasy consumption and patients with acute hepatitis

B virus (HBV) infections due to careless sexual practices have full access to LT waiting lists raises the question as to why patients with severe acute AH or acute-on-chronic liver failure should be treated any differently[44]. The lack of pre-LT abstinence should not be considered as a justification for denying the legal right of patients with advanced ALD to have access to LT waiting lists[45]. The sidelining of patients with severe AC who, after complete evaluation, are otherwise considered to be candidates for LT must be avoided[46]. There are no moral or ethical arguments that could justify the exclusion of very ill patients with ALD from potentially lifesaving LT, as exclusion could be considered a death sentence for these patients[20,47].

ALD diagnoses are often made in the later stages of the disease because patients often remain in primary care for a long time, managing their alcoholism, and ALD diagnosis only occurs when hepatic manifestations belatedly raise clinical suspicion. Poor patient awareness, misinformation among the referring clinicians, delayed alcohol cessation intervention and counseling, premature and overconfident attribution of liver disease to another etiology (*e.g.*, HBV or HCV) are just some of the factors that limit effective management of AC[48,49].

In the end, alcoholism has to be accepted as a disease that, in some cases, has a genetic background[50]. As alcoholism is a life-long disease, it is to be expected that it should persist after LT[51].

LT FOR ALD

ALD is a worldwide health problem, resulting in high morbidity and mortality, not only due to the effects of alcohol on the liver, but because of the risk it poses to the health of other organs and the increased risk of accidents and violence-related deaths[52]. One type of ALD is AC, which is a leading indication for LT in the Unites States and Europe, accounting for approximately 15% and 20% of LT cases, respectively[53-55]. A recent analysis of three US databases (the National Health and Nutrition Examination Survey, HealthCore, and UNOS) showed that the proportion of patients on the LT waiting list or the proportion who have undergone LT due to cirrhosis secondary to HCV infection is declining, while the proportion on the list or who have undergone LT due to non-alcoholic fatty liver disease or ALD is increasing[56,57]. These findings are probably due to the advent of highly effective and well-tolerated treatments for HCV infection, which, up until now, was the main indication for LT[52].

PRE-LT EVALUATION OF PATIENTS WITH ALD

Comorbidities
In general, the indications and contraindications for LT in patients with AC are the same as those for patients with cirrhosis of any etiology[52]. There are, however, specific factors that should be addressed when evaluating AC

patients for LT, given that they have a high prevalence of multisystemic alcohol-related changes. These comorbidities can be neurological (dementia, peripheral neuropathy, and vertigo), cardiological (cardiomyopathy, hypertension, and chronic renal disease), hematological (chronic anemia), gastrointestinal (chronic pancreatitis, diarrhea, and malnutrition), musculoskeletal (sarcopenia and osteoporosis), or psychiatric (including tobacco and illicit substance use)[57-61]. For example, malnutrition is present in about two-thirds of patients with cirrhosis on the LT waiting lists and negatively impacts survival, quality of life, and the patient's ability to cope with surgery or infections. When alcohol is an etiologic factor underlying cirrhosis, the prevalence of malnutrition is higher[62,63]. The incidence of comorbidities in AC has recently been reviewed, and a high comorbidity rate was found [hazard ratio (HR) for any comorbidity: 3.74; 95%CI: 3.56-3.94], including for non-cancer comorbidities (HR for any non-cancer comorbidity: 4.33; 95%CI: 4.06-4.62), but with the exception of acute myocardial infarction. The presence of these comorbidities must be carefully evaluated before LT, as they may negatively impact LT outcomes[57,64].

Alcohol consumption progresses over the years, and alcohol and its metabolites are toxic *per se*. However, there are other mechanisms of action regarding the negative health effects of alcohol consumption. By increasing gut permeability and exposing Kupffer cells to Gram-negative intestinal bacteria, alcohol induces cytokine production and a systemic inflammatory response[65].

An additional challenge of LT for AC is the need for lifelong post-LT follow-up, taking into consideration the comorbidities that may not be fully resolved or may return (along with alcohol consumption relapse) in the post-LT period. It is not surprising that cardiovascular illnesses and de novo malignancies are significantly over-represented in AC patients who underwent LT[53]. With the recent advances in hepatitis C treatment and lack of HCV-related LTs, long-term follow-up of LT recipients will often entail more challenging circumstances involving patients who underwent LT for non-alcoholic steatohepatitis or ALD.

Neurological comorbidities
Chronic heavy intake of alcohol is a well-established cause of brain atrophy and dementia[54]. Patients may present with mild-to-moderate short- or long-term memory issues or more severe manifestations. There may be deficits in attention, concentration, learning, abstract reasoning, and motor skills. Hepatic encephalopathy can prevent a proper neurological evaluation of alcohol-induced brain damage prior to LT. These clinical manifestations may or may not be fully reversible after alcohol cessation. After LT, neurological improvement in AC patients with encephalopathy is not as good as for patients with cirrhosis of a different etiology[37].

Wernicke's encephalopathy is an alcohol-related syndrome characterized by ataxia, ophthalmoplegia, and

confusion, often with associated nystagmus, peripheral neuropathy, cerebellar signs, and hypotension. There is impaired short-term memory loss and emotional lability. Wernicke-Korsakoff's syndrome can develop after Wernicke's encephalopathy, characterized by anterograde and retrograde amnesia and confabulation. Wernicke-Korsakoff's syndrome is caused by a chronic thiamine deficiency, resulting in damage to the thalamic nuclei, mammillary bodies, brainstem, and cerebellar structures.

Alcohol can cause a polyneuropathy that can involve paresthesia, numbness, weakness, and chronic pain. Other neurologic conditions associated with chronic alcohol consumption are headache (cluster and migraine), neurocardiogenic (vasovagal and vasodepressor) syncope, compromised olfactory function, sleep disturbances, and peripheral vertigo. A small proportion of patients (< 1%) may develop midline cerebellar degeneration with ataxia. Seizures occur frequently upon alcohol withdrawal[66].

Cardiovascular comorbidities

The most common complications of ALD are cardiomyopathy, hypertension, and supraventricular arrhythmias. Alcoholic cardiomyopathy is the most common type of non-ischemic cardiomyopathy in Western countries (approximately 45% of cases). When being evaluated for surgery, many patients with ALD are found to have asymptomatic cardiac involvement, and are at risk of adverse short- and long-term outcomes. These findings may be confused with the findings associated with cirrhotic cardiomyopathy[53]. The clinical manifestations of ALD are similar to other causes of cardiac failure.

Abstinence can result in improvement in some cases. There are findings of beneficial cardiovascular effects with moderate alcohol consumption, but, when alcohol consumption is excessive, it results in hypertension. It has been shown that reducing alcohol dose-dependently decreases blood pressure, especially in heavy drinkers, and hypertension disappears with ⩽ 2 doses/d[37]. Chronic alcoholism is associated with a higher risk of cardiovascular mortality due to its epidemiological associations with known risk factors (smoking, age > 50 years, dyslipidemia, obesity, and hypertension).

The most common arrhythmias related to alcohol consumption are atrial fibrillation and supraventricular tachycardia, which commonly occur during acute intoxication and withdrawal. Cardiomyopathy can also induce ventricular arrhythmias.

Gastrointestinal comorbidities

Acute and chronic alcohol consumption cause mucosal inflammation, impairment of gut motility, sphincteric dysfunction, increased acid output, and damage to the small intestinal mucosa; these issues can occur directly due to a toxic effect or indirectly due to bacterial overgrowth and an impaired immune response[67]. Disregarding cirrhosis-related varices, Mallory-Weiss tears are a major cause of gastrointestinal bleeding,

and a history of alcohol use can be found in > 40% of cases[68]. Diffuse esophageal spasm is also more frequent in individuals with alcoholism.

Alcoholic gastropathy (submucosal hemorrhages) typically involves abdominal pain, nausea, and vomiting.

Alcoholism accounts for about one-third of all cases of pancreatitis. The risk of pancreatitis in patients with alcohol dependence is approximately 4-fold higher than that in the general population, and it increases according to dose. Chronic pancreatitis develops in 10% of alcohol addicts after 6-12 years of 80 g daily alcohol intake[69]. Individuals with recurrent acute episodes of pancreatitis may develop chronic pancreatitis that can aggravate malnutrition.

Hematopoietic system comorbidities

The anemia that is commonly seen in patients with chronic alcohol problems can be multifactorial. Blood loss can cause anemia due to iron deficiency, which can occur due to the gastrointestinal diseases mentioned above. Dietary folate deficiency can cause megaloblastic anemia. Alcohol also has a direct toxic effect on the bone marrow, which can lead to sideroblastic anemia that resolves after abstinence. Alcohol also suppresses megakaryocyte production causing thrombocytopenia, which rapidly resolves about a week after cessation of alcohol intake. Lastly, alcohol interferes with platelet and white blood cell function, increasing the risks of bleeding and particular infections[66].

Malignancies

Previous alcohol abuse was shown to be associated with a 3-fold increased risk of post-LT *de novo* tumors. The mean duration until diagnosis has been reported to range between 3 and 5 years after LT[37]. Chronic alcohol use increases the risk of head and neck cancer, squamous cell carcinoma of the esophagus, and breast, prostate, pancreas, cervix, lung, and colon cancer. The risk is further potentiated by concomitant smoking and remains elevated despite alcohol abstinence, so screening should be considered after LT[66].

Infectious diseases

It has been demonstrated that both chronic alcohol consumption and moderate acute drinking can modulate the function of cells of the innate immune system, such as monocytes, macrophages, and dendritic cells. Alcohol is associated with increased intestinal permeability to endotoxins, altered proportions of monocyte population subsets, and altered cytokine profiles. These changes subside after 14 d of abstinence[70]. Alcoholism is associated with increased frequency and severity of infections, such as epidural abscesses, tuberculosis, meningitis, pneumonia, tick-borne fever, and others.

Psychiatric comorbidities

Psychiatric illnesses are commonly associated with alcoholism. The psychiatric and social issues associated with alcoholism can be more severe than the direct

medical effects. Anxiety and other mood disorders are found in at least a third of patients with alcoholism and multiple drug use is also prevalent. This is considered to be a bidirectional relationship. Alcohol may be used as a "medication" to relieve symptoms and, on the other hand, chronic use may lead to the development and/or worsening of these symptoms, either by compromising social skills or through the direct effect of alcohol on the brain[71]. Alcohol-related behavioral issues can cause other health issues, including domestic abuse injuries, other violence-related trauma, motor vehicle accidents, and burns.

Other diseases

There are many other conditions associated with alcohol consumption, including rhabdomyolysis, osteonecrosis (avascular necrosis), IgA nephropathy, and porphyria cutanea tarda.

ALD, HCC, AND SURVEILLANCE

In AC patients, mainly in those who were drinking > 80 g of alcohol daily, there was a positive association between the amount of alcohol intake and the risk of HCC (HR: 4.5), and this increased by 22% for those who drank 6 alcoholic units/day. In countries where there is heavy alcohol consumption, the cumulative risk of HCC is increased by 5-7-fold[72-74]. Several recent studies have established that the underlying etiology of liver disease determines the cumulative risk of HCC, and patients with viral, fatty liver, or autoimmune cirrhosis have a higher risk than those with AC[75-77].

The HCC surveillance recommendations for AC patients are similar to those for other cirrhotic patients (*i.e.*, periodic 6-mo ultrasound screening), with no specific recommendations. In alcoholic patients, the risk factors are age, metabolic syndrome, and the severity of the underlying liver disease[78]. In a surveillance study of 450 AC patients [Child-Turcotte-Pugh (CTP) classes A and B], Mancebo *et al*[79] found that 62 patients developed HCC, with an annual incidence of 2.6%. The risk was independently associated with age (> 55 years) and platelet count (< 125000/mm³). The annual incidence was 0.3% in patients without risk factors, 2.6% in patients with one risk factor, and 4.8% in patients with two risk factors (*P* < 0.0001). Genomic analysis of alcohol-related HCC has demonstrated the presence of mutations in genes that modulate the HCC pathway, which helps to better define the risk classes and to adapt strategies for HCC surveillance.

NUTRITIONAL EVALUATION IN ALD

Poor nutritional status in patients with liver diseases is common, occurring in 20%-90% of cases. The main outcome is loss of muscle mass and fat, which is associated with the etiology of liver disease[80-83]. In ALD, there is marked loss of weight and muscle mass, with

deficiencies of macronutrients and micronutrients, which can adversely affect the body composition of AC patients[84,85].

High daily alcohol intake can end up ensuring caloric maintenance, despite the fact that excessive alcohol consumption can inhibit hunger and compromise the palate, stimulating the search for ultra-processed foods, which also compromise the body composition of this population[86].

The change in muscle mass in quantity and/or function characterizes a clinical condition called sarcopenia. In some cases, no weight loss is observed. However, a discrepancy between the percentages of lean and fat mass may occur, which determines the diagnosis of sarcopenic obesity[86]. In sarcopenic obesity, there are mitochondrial and bioenergetic dysfunctions. The neurological consequences of alcohol that generate fatigue and asthenia make it difficult to determine whether changes in the skeletal muscles are the direct effects of ethanol and/or ALD.

After LT, some of the complications of cirrhosis disappear, but this does not occur easily with sarcopenia; on the contrary, the complications may be aggravated by the use of post-LT immunosuppressants[87,88]. Clinical improvement is not directly proportional to muscle function and muscle turnover but, in ALD, sarcopenia worsens the prognosis. The mechanisms involved are still unclear[89].

Nutritional assessment in patients with liver diseases has limitations due to difficulties with reproducibility and the lack of a gold standard. The current most accurate assessment involves the use of several methods that may be complementary[90]. The classic anthropometric assessment involving the assessment of body mass index (BMI), arm circumference, arm muscle circumference (BMC), and tricipital skinfold is a low-cost, universally used nutritional assessment. However, it has poor reproducibility (regarding inter- and intra-observer assessment) and it does not accurately measure muscle and fat mass in cases of cirrhosis involving ascites and edema[91].

The Subjective Global Assessment (SGA) is a low-cost, easy to apply method. However, it has flaws in implementation. It is based on body weight and subjective assessment and involves data that are self-reported by the participant (or guardian), with no accurate quantification of muscle mass[92-96]. The Patient-Generated Subjective Global Assessment (PG-SGA) is being used routinely, with early nutritional risk being identified more accurately, taking into account the disease staging and the patient's drug regimen; however, this method has only been validated for cancer patients[97].

The determination of the function of cirrhotic muscle mass using dynamometry is low cost and reproducible. However, it may not reflect the patient's actual nutritional status, especially in ALD patients, as it is based on the principle of muscular contractility and the use of alcohol

(depending on the amount of alcohol and duration of abstinence) may compromise these results. In AC patients with encephalopathy, it is not possible to apply this method because it is based on the principle of hand-grip strength[90,97-99].

Methods for quantifying lean mass, such as bio-electrical impedance analysis (BIA), dual energy X-ray absorptiometry (DEXA), and impedance plethysmography, are reproducible and objective. However, BIA and plethysmography may be inaccurate in cases of "body asymmetry" (*e.g.*, involving ascites and edema) and DEXA is a potential radiation-related risk factor (especially if it is routinely used) and is expensive, making it difficult to use in clinical practice[100-102].

Imaging methods, such as computed tomography (CT) and magnetic resonance imaging (MRI), can be used to quantify skeletal muscle mass, but they are high-cost methods[103,104]. The skeletal muscle area determined from a single CT or MRI section involving the third or fourth lumbar vertebra has been shown to reflect the muscle mass of the entire body[103]. Using these methods, patients with cirrhosis have lower muscle and fat mass compared to controls[87,105]. AC patients have been included in several studies but etiology-specific data have not yet been reported. Ultrasound assessments of lean muscle mass exhibit variability across observers, and analyzing body portions only provides an estimate of whole-body measurements and does not allow assessment of muscle functionality[87,104,105].

The above-mentioned methods all have limitations because they are based on the body composition model, valuing only lean muscle and fat mass. In 2000, Ellis proposed the use of a cellular composite multicompartment model of body composition[106]. This evaluates cellular functionality and can aid in an objective, reproducible, and serial way in the determination of cellular composition and functionality based on the use of the BIA phase angle (PA). PA is based on measurement of electrical resistance (R) and reactance (Xc) and it reflects the structure and functionality of the cell membrane[107].

Studies involving different populations have identified PA cutoff points that differentiate the pathophysiology in question from a lack of the pathophysiological condition. ALD patients have different cellular characteristics than patients with other conditions. This is because ethanol induces autophagic mechanisms (adaptive cellular responses to eliminate damaged organelles) and the production of cytotoxic cellular proteins (due to changes in homeostasis, protein synthesis, and autophagic proteolysis) that result in the loss of skeletal muscle[108]. Factors that contribute to cellular damage in ALD patients include hyperammonemia (due to cirrhosis), endocrine abnormalities (such as hypogonadism), and intestinal dysbiosis.

PA assessment of cirrhosis prognosis has a sensitivity of 68.9% and a specificity of 70.0%, indicating that it is a good prognostic marker. Assessing PA to investigate different cirrhosis stages is useful, as a lower PA indicates a worse disease stage[90,109-112]. In the follow-up of pre- and post-LT patients, Deutrich *et al*[113] observed that changes in PA were the only measurable changes that correlated with the improvement in the clinical condition. Two studies evaluating cirrhotic patients identified the same cutoff point for PA (5.4°), where those who were below this value had a poor prognosis[90,114]. Recently, a pilot study of cirrhotic patients identified a cutoff of 4.9°[115], which is different from the previous study findings. This demonstrates the need for cohort studies with greater robustness in order to determine a reliable cutoff point that can indicate the prognosis/clinical condition of cirrhotic patients.

For Baumgartner *et al*[116], the PA is an indicator for the diagnosis of metabolic, physiological, nutritional, and hydration disorders that could be applied to any living creature. In an experimental study of rats with carbon tetrachloride (CCl_4)-induced cirrhosis, PA was determined before and after induction of cirrhosis, being reduced in cirrhotic animals. It was also observed that a decrease in fatty acids (FA) accompanied the worsening of the cirrhosis, measured by cellular damage using the thiobarbituric acid reactive substances (TBARS) technique, compared to controls[117].

In different liver diseases, PA can provide different relevant information. For example, in chronically infected HCV patients, PA is a good predictor of advanced fibrosis; each degree of decrease in FA increases the risk of advanced fibrosis 4-fold. In HCV patients undergoing antiviral treatment, the reduction in PA is associated with an increase in the adverse effects of the therapy[118,119].

PA also serves as a basis for bioelectrical impedance vector analysis (BIVA), a slightly more complex evaluation, which provides information on the body composition (cellularity) and cell hydration state, independently of the alteration in body composition. BIVA is of great importance in cases of edema and ascites that make identifying nutritional compromise difficult[120,121].

There have been no studies of PA and/or BIVA in patients with ALD alone. There is a need to develop this research area further to understand cellular functioning in ALD patients in order to develop preventive and curative strategies regarding nutritional and other clinical issues, improving quality of life and post-LT outcomes.

FUNCTIONAL LIMITATIONS IN CIRRHOTIC PATIENTS

Cirrhosis leads to systemic and metabolic alterations that compromise pulmonary, renal, encephalic, cardiac, and metabolic functions, and complications such as ascites, encephalopathy, jaundice, and sarcopenia, which increase morbidity and mortality and compromise quality of life[122,123].

Metabolic changes associated with cirrhotic malnutrition are frequent, negatively affect the musculoskeletal system, and directly interfere with physical fitness[124,125]. The deficiency in protein synthesis leads to persistent cachexia, which limits the physiological integrity of the

Table 1 Liver transplantation survival rates reported by the European Liver Transplant Registry

n	Etiology	1 yr	5 yr	10 yr
		%		
15019	AC	86	73	59
1790	AC + HCV	85	69	54
6507	Acute liver failure	70	64	58
10753	HCV	80	65	53
4187	HBV	83	74	68
9122	Cirrhosis + HCC	83	62	49
9114	Cholestasis	87	78	70
1892	AIH	85	76	67
468	Hemochromatosis	76	66	53

AC: Alcoholic cirrhosis; HCV: Hepatitis C virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis.

muscular system and impairs its functioning, with loss of muscle strength and worsening of quality of life. Protein filaments of actin and myosin undergo adaptive processes and lose their contractile components, with muscular hypotrophy. Concomitantly, there is a change in lipid metabolism that influences the biochemical composition of lipoproteins. In cirrhotic patients, there is insufficient glycogen due to limitations in hepatic synthesis, which increases the use of amino acids as an energy source and causes an acceleration in the decomposition of skeletal muscle to release amino acids, which results in a loss of muscle mass. These changes limit muscle functioning and negatively affect the performance of daily activities[126].

A possible explanation for the reduction in function may be related to the loss of muscle mass in this population, but it may also be due to a decrease in the mitochondrial oxidative capacity and/or number of mitochondria in the muscle tissue. The adenosine triphosphate (ATP), phosphocreatine, and total magnesium (Mg^{2+}) levels are decreased in cirrhotic skeletal muscle. This concept was demonstrated by Jacobsen *et al*[127], who found higher rates of mRNA and mitochondrial ATP in patients with CTP cirrhosis compared to those with CTP-B and CTP-C scores (Table 1).

Sarcopenia is associated with age, but it is also present in chronic neoplastic diseases and leads to a decrease in functional capacity and an increased risk of mortality[128]. Severe muscle depletion or sarcopenia is defined as a decrease in muscle mass, strength, and function. Sarcopenic dysfunctions are predictors of morbidity and mortality in cirrhotic patients[129].

Abdominal cross-sectional studies [involving lumbar segments 3 and 4 (L3-L4)], including those that involve CT or MRI, represent the gold standard for quantifying skeletal muscle mass. They involve detailed objective assessment for the identification of sarcopenia and assist in improving the nutritional/metabolic outcomes of patients with cirrhosis[130]. Muscle tissue loss may be an important factor for muscle dysfunction and worsening of quality of life. However, it is necessary to specifically measure muscle dysfunction with precise methods, such

as isokinetic dynamometry, palmar grip dynamometry, and manovacuometry (which measures respiratory muscle strength)[131].

Montano-Loza *et al*[130], using measurements based on abdominal L3-L4 CT images, evaluated the impact of sarcopenia on cirrhosis in patients on a waiting list for LT. They found a 6-mo survival rate of 71% in sarcopenic patients and 90% in non-sarcopenic patients. The frequency of sepsis and death was significantly higher in the sarcopenic patients[130].

Regarding functional evaluation, the 6-min walk test (6MWT) is an accessible, easy, cheap, and reproducible assessment. In cirrhotic patients, 6MWT performance < 400 m is an independent predictor of mortality. Based on a receiver operating characteristic (ROC) curve analysis, 6MWT had a greater sensitivity and specificity for mortality compared to maximal oxygen consumption and respiratory muscle strength[132].

The muscular dysfunctions in patients with cirrhosis may be influenced by the etiology of the disease. Patients with AC may present with alcoholic myopathy. Galant *et al*[133] showed that AC patients had lower muscle strength and poorer 6MWT performance and quality of life compared to cirrhosis patients with HBV or HCV. Physical fitness is a marker of mortality due to cirrhosis in AC patients, with patients with a peak oxygen uptake (VO_2) < 14 mL/kg having a lower survival rate over 3 years compared to those with superior results.

A multidisciplinary intervention for muscular recovery in patients with cirrhosis, involving nutritional supplementation and supervised physical activity, led to a gain in muscular mass and improvement in functional activity, directly impacting quality of life and preparation for LT (Figure 1)[134].

MANAGEMENT OF ALCOHOL ADDICTION BEFORE LT

The patient's history of alcohol and other substance use, such as tobacco, opioids and illicit/recreational drugs must be thoroughly evaluated[52]. Psychiatrists, psychologists, social workers and dependency specialists are essential in the evaluation of these patients. The information collected will help the multidisciplinary team to determine whether a transplant should be performed in these patients, as well as to establish a therapeutic plan before and after the procedure[52,135,136]. There is evidence that the work of such teams in transplant centers reduces the rates of recurring alcoholism and mortality after LT compared to patients referred for outpatient treatment[55,60]. Simple standardized questionnaires, such as CAGE and Alcohol Use Disorders Identification Test (AUDIT), can be used in clinical practice to track the chronic and excessive use of alcohol in transplant patients, including those with cirrhosis of other etiologies[52,137,138]. An additional tool for the psychosocial assessment of transplant candidates is the Stanford Integrated Psychosocial Assessment for Transplantation. Its strengths include standardization
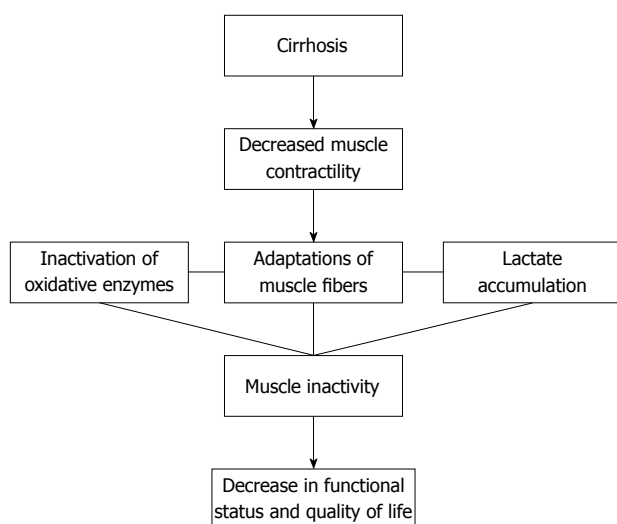
Figure 1 Flow chart demonstrating the consequences of cirrhosis regarding muscular adaptation and functional repercussions[134].

of the evaluation process and the ability to identify individuals at risk of negative psychosocial events after transplantation in order to develop interventions aimed at improving the patient's pre-LT conditions. This instrument also allows evaluation of the psychosocial factors that best predict patient compliance and graft survival. Eighteen risk factors are divided into four domains, including patient readiness, social support, psychological stability and substance use. Based on an index composed of all the domains, patients are classified as excellent, good, minimally acceptable, high risk or poor transplant candidates[52,139].

A constant concern of health professionals is the patient's possible return to alcohol use after LT or the relapse of an ALD patient before transplantation[40]. To date, however, no standardized definition of recurrence has been formulated. Consequently, the rates found in the literature vary widely, due both to the different definitions of the term and different follow-up times. A 2015 review[17] indicated rates from 3% (ingestion > 60 g/d) to 43.70% (any consumption). It seems important, then, to distinguish between patients who occasionally drink small amounts of alcohol from those who regularly drink moderate amounts from those who continuously drink large amounts. Thus, recurrence can be distinguished from alcoholism[136]. Nevertheless, it requires emphasis that patients who underwent LT for ALD are obligated to stay abstinent. Unfortunately, this is an extremely difficult goal to achieve consistently.

Predictive factors of recurrence
In 1997, an American consensus suggested that patients with AC should have a minimum abstinence period of 6 mo before being included on the LT waiting list, the so-called "6-mo rule." The rationale of this recommendation was to evaluate the improvement of liver function, commonly observed after three to 6 mo of sobriety[140,141]. This abstinence period also serves as

a predictor of post-transplant recurrence. As a result, major guidelines recommend this rule[55,135]. The logic is that the longer the period of sobriety, the lower the risk of returning to alcohol use. However, rules based on specific sobriety times, particularly those of short duration, are not consistent with what is known about the evolution of alcohol use disorder or predictions of future abstinence[142,143]. Although each month of sobriety increases the likelihood that the patient will not resume drinking, patients abstinent for 6 mo have only a slightly better risk reduction than those with 4 or 5 mo of sobriety[60]. Therefore, rigid adherence to the "6-mo rule" could result in unnecessary delays in listing patients who would otherwise be good candidates for transplantation, especially if we consider that this abstinence period is a weak predictor of post-transplant alcohol consumption[52]. For this reason, other predictors of recurrence after LT should be identified besides a specific period of sobriety. A 2015 review[21] highlighted an association between the following factors and recurrence: psychiatric co-morbidity, poor social support, multiple treatment failures, illicit drug use, a family history of alcoholism, medical non-compliance and the continued use of alcohol despite its consequences. It is up to the multidisciplinary team to investigate the presence of these factors and, if appropriate, adopt the best strategies for circumventing them. However, other factors suggest a lower risk of recurrence, such as: the patient's recognition that alcoholism is a disease, family support, employment, having a permanent residence, the ability to perform activities that replace daily drinking, and participation in rehabilitation programs[21,52].

LT SURGICAL ISSUES IN ALD

At present, there is no evidence for the association between ALD and increased incidence of portal vein or arterial thrombosis pre- or post-LT. The type of LT technique used is based on the experience of the transplant center, and there are no recommendations based on cirrhosis etiology. The techniques include the piggyback technique (preservation of the vena cava with lateral-lateral cava-cava anastomosis or suprahepatic cava-hepatic veins terminal-terminal anastomosis) and conventional vena cava-cava terminal-terminal anastomosis (without preservation of the recipient's vena cava).

NEED FOR SURGERY AFTER LT

Causal associations have been established between alcohol consumption and cancers of the oral cavity, pharynx, larynx, esophagus, liver, colon, rectum, and, in women, breast; associations are also suspected for cancers of the pancreas and lung[26,37,136,144,145]. Evidence suggests that the effect of alcohol is modulated by polymorphisms in genes encoding enzymes for ethanol metabolism, folate metabolism, and DNA repair. The mechanisms by which alcohol consumption exerts a

carcinogenic effect have not been completely defined[145].

The higher risk of malignancies for patients with AC should be considered in the routine assessment of these patients[37]. Surveillance protocols for earlier detection of *de novo* malignancy are needed to improve long-term post-LT outcomes[144]. Appropriate surgical treatment is the best option for curing solid organ malignant neoplasms, such as skin, esophageal, lung, digestive, or head and neck cancer. Proper resection of these lesions can contribute to increased survival when the disease is diagnosed in the early stages. Radiotherapy, chemotherapy, or immunotherapy are indicated for palliative care in the advanced stages of each specific neoplastic disease.

ACUTE AH

Background

AH is a distinct severe form of steatohepatitis that occurs in patients with alcoholism. It can present as acute or chronic liver failure associated with a rapid decline in liver synthetic function, and a consequent increase in mortality[146,147]. The mortality rate in patients with severe AH is 30%-50% at 3 mo, often despite supportive medical care. The amount of alcohol intake that puts an individual at risk of AH is not known, but generally, most AH patients have a history of heavy alcohol use (> 100 g/d) for decades[35,146-149].

The pathogenic pathways that lead to the development of AH are complex and involve oxidative stress, gut dysbiosis, and dysregulation of the innate and adaptive immune system, with injury to parenchymal cells and activation of hepatic stellate cells[146].

Incidence

The annual incidence of AH remains largely unknown. Concerning its prevalence, a large study of systematic biopsies in 1604 alcoholic patients, symptomatic or not, showed the prevalence of AH to be 20%[149]. In symptomatic patients, including those with decompensated liver disease, the prevalence of AH is not well known, partly because most centers rely on clinical criteria and do not consider transjugular liver biopsy as a routine practice in the management of patients with decompensated ALD[35].

History

Patients with AH are often aged 40-50 years, with most patients presenting before the age of 60 years. Patients with AH typically have a history of daily heavy alcohol use (> 100 g/d) for > 20 years[150].

Clinical features and diagnosis

The characteristic clinical features of AH are malaise, anorexia, fever, jaundice, tender hepatomegaly, signs of malnutrition, and complications such as ascites or variceal bleeding[146,151]. Progressive jaundice is the main presenting feature of symptomatic cases.

Patients with severe AH and/or underlying AC may exhibit signs of hepatic encephalopathy, may develop hepatorenal syndrome, and are prone to developing bacterial infections[147]. Serum aminotransferases are moderately elevated (typically < 300 IU/L and rarely > 500 IU/L), with aspartate transaminase (AST) > alanine transaminase (ALT) and often > 2:1, which is rarely seen in other forms of liver disease[152-154]. Patients with AH typically have elevated serum bilirubin and γ-glutamyltransferase (GGT) and leukocytosis with a predominance of neutrophils. Depending upon the severity, serum albumin may be decreased, and the international normalized ratio (INR) may be elevated[35].

Imaging tests and liver biopsy

Abdominal imaging (ultrasound, CT, and MRI scans) in patients with AH may suggest fatty changes in the liver, evidence of underlying AC, or ascites. Transjugular liver biopsy is recommended, as about 30% of patients diagnosed with AH can be misdiagnosed when the diagnosis is based only on clinical parameters[155]. Histologic findings in liver biopsies from patients with AH include steatosis (typically micro- or macrovesicular steatosis, but in some cases alcoholic foamy degeneration is seen); hepatocellular ballooning with cytoplasmic rarefaction, Mallory-Denk bodies; neutrophil or lymphocyte infiltration; cholestasis and bile duct proliferation; and fibrosis with a perivenular, perisinusoidal, and/or pericellular distribution[156,157].

Determining disease severity

Several models have been proposed to determine the severity of AH and predict early death 1-2 mo after hospitalization[35]. The Maddrey discriminant function (DF) and the MELD score are the most commonly used scores to help identify patients who are more likely to benefit from pharmacotherapy. Other validated scores include the Glasgow Alcoholic Hepatitis score, ABIC score (which includes Age, serum Bilirubin, International Normalized Ratio, and serum Creatinine), and Lille score (which is used to determine whether a patient is responding to treatment)[35,158,159].

Maddrey DF: The Maddrey DF (also known as the Maddrey score) was the first score to be developed and remains the most widely used. It is calculated as follows[160,161]:

DF = {4.6 × [prothrombin time (s) - control prothrombin time (s)]} + [serum bilirubin (mg/dL)]

In the absence of treatment, the 1-mo spontaneous survival of patients with a DF $\geqslant$ 32 has fluctuated between 50% and 65%[162,163]. Those with lower scores have low short-term mortality rates and do not appear to benefit from glucocorticoids[164].

MELD score: The MELD score is a statistical model developed to predict survival in patients with cirrhosis that has also been used to predict mortality in patients

hospitalized for AH[165,166]. The score ranges from 6 to 40 and is based on serum bilirubin, creatinine, and INR.

In one report, a MELD score > 11 performed as well as the DF in predicting 30-d mortality[165]. The sensitivity and specificity of MELD for predicting 30-d mortality was 86% and 81%, respectively, and 86% and 48%, respectively, for the DF. In a second study, a MELD score $\geqslant$ 21 had a sensitivity of 75% and a specificity of 75% for predicting 90-day mortality[167]. In addition, an increase in the MELD score $\geqslant$ 2 points in the first week of hospitalization may independently predict in-hospital mortality[166].

Lille score: The Lille score is a method to determine whether patients with AH are responding to glucocorticoid therapy. It combines six variables: age, renal insufficiency (creatinine > 1.3 mg/dL or creatinine clearance < 40 mL/min), albumin, prothrombin time, bilirubin, and change in bilirubin at day 7 (bilirubin at day 7 - bilirubin at day 0)[165]. The Lille model guides treatment decisions, with a score > 0.45 suggesting that a patient is not responding to glucocorticoids and predicting a mortality rate of 75% at 6 mo[158]. Based on the Lille score, corticosteroid treatment can be stopped in those with no improvement after a week of therapy[168].

General management
Patients with AH require general supportive care, including support for alcohol abstinence, prevention and treatment of alcohol withdrawal symptoms, fluid management, nutritional support (enteral feeding is preferred over intravenous nutrition), correction of nutritional deficiencies, infection surveillance, prophylaxis against gastric mucosal bleeding, and discontinuation of nonselective beta blockers in patients with severe AH (in addition, beta blockers, if indicated, should not be started in patients with AH until after they have recovered)[164,168,169].

Infections are frequent and difficult to diagnose in AH patients as they often fulfil the criteria for systemic inflammatory response syndrome (SIRS) at admission, which reflects either the inflammatory state associated with the AH episode or an ongoing bacterial infection. Systematic body fluid sampling and close clinical monitoring are advised for early detection of infection. In the absence of scientific evidence, criteria for initiating empirical antibiotic administration, although widely used, remain debated. In patients with severe AH, infection screening at admission is particularly warranted because a quarter of them have infections at admission[170].

Mild to moderate AH
Patients with mild to moderate AH (Maddrey DF < 32) and without corticosteroid treatment have only a 10% mortality rate at 28 d. Supportive management is therefore adequate for such patients[171]. The mainstay of treatment for patients with mild to moderate AH is abstinence from alcohol. In addition, general supportive

care (*e.g.*, nutritional support and hydration) should be provided, but pharmacological treatment with glucocorticoids is not recommended as it does not appear to be beneficial in patients with mild to moderate AH. Pentoxifylline has only been studied in patients with severe AH and not in patients with mild to moderate AH[164].

Severe AH
In addition to general supportive care, pharmacological treatment is indicated for patients with severe HA (Maddrey DF $\geqslant$ 32). Most guidelines recommend treating patients with severe AH with prednisolone at 40 mg/d for 4 wk (then tapering the dose over 2-4 wk, or stopping prednisolone treatment, depending on the clinical situation), provided there are no contraindications for its use (*e.g.*, active bacterial or fungal infection or chronic hepatitis C or B)[35,172]. Steroids have a potent immunosuppressant effect, suppressing two pro-inflammatory transcription factors: nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1). This results in lower levels of tumor necrosis factor (TNF)-α and interleukin (IL)-8. In a randomized trial [STeroids Or Pentoxifylline for Alcoholic Hepatitis (STOPAH)] involving 1103 patients with severe AH, prednisolone showed a trend toward improving survival (odds ratio: 0.72; 95%CI: 0.52-1.01)[173].

Pentoxifylline is an oral phosphodiesterase inhibitor that also inhibits the production of TNFα, which is increased in patients with AH, among other cytokines. The role of pentoxifylline in the treatment of AH remains uncertain because questions remain regarding its efficacy[173-175]. Pentoxifylline may be an alternative in patients who are at risk of sepsis or failing to follow up after discharge (thereby making tapering off prednisolone unlikely, which could result in serious adverse effects). Pentoxifylline is given as a 400 mg dose three times/day (or 400 mg once/day in patients with a creatinine clearance < 30 mL/min)[164]. The largest meta-analysis published on this topic supports the observation that pentoxifylline decreases the risk of acute kidney injury and suggests that pentoxifylline improves the mortality rate compared with placebo but not compared to glucocorticoids[174]. The American Association for the Study of Liver Diseases and the European Association for the Study of the Liver recommend pentoxifylline for patients who cannot receive glucocorticoids[35,172].

A multicenter randomized placebo-controlled trial of prednisolone, pentoxifylline, and combination therapy has been completed. It showed that 4-week treatment with combination therapy compared with prednisolone alone did not improve the 6-mo survival rate[176].

Prognosis
Mortality rates among patients who do not receive pharmacological therapy (*e.g.*, prednisolone) for AH are variable. In patients with severe AH, short-term mortality rates are high (approximately 25%-45% at 1 mo)[160,177-179], whereas patients with mild to moderate

AH have lower short-term mortality rates (< 10% at 1-3 mo)[180,181]. Multiple risk factors for increased mortality in patients with AH have been identified and some have been incorporated into prognostic models. These include older age, acute kidney injury, elevated bilirubin levels, elevated INR, leukocytosis, alcohol consumption > 120 g/d, infection (sepsis, spontaneous bacterial peritonitis, pneumonia, and other infections), hepatic encephalopathy, upper gastrointestinal bleeding, bilirubin to GGT ratio of > 1, and meeting the criteria for SIRS[164].

Long-term outcomes after initial hospitalization for AH

An important determinant of the outcome among patients with AH is whether they continue to drink alcohol. In a case series involving 87 patients with AH who survived their index hospitalization, the overall estimated 5-year survival rate was 32%. However, among those who abstained from alcohol, the estimated survival rate was 75%, whereas for those who relapsed and continued to consume alcohol it was 27% and 21%, respectively[182].

LT IN AH

AH is a clinical syndrome associated with hepatic impairment and systemic inflammatory response that is observed in alcoholic patients (most of them cirrhotic). It is characterized by progressive jaundice, mild to moderate elevation of liver enzymes, coagulopathy and hepatic encephalopathy[52,155]. In its severe form, mortality is 30% to 50% at 3 mo and up to 70% at 6 mo, especially when associated with renal impairment[147,176]. Most transplant centers adopt the "6-mo rule" before listing patients with ALD. However, patients with AH that do not respond to clinical treatments cannot wait 6 mo to be placed on an LT waiting list, considering the short-term mortality rate, which can be as high as 50%[183]. Thus, the lack of salvage treatment for these patients is the basis for considering immediate LT.

In a case-control study, Mathurin *et al*[184] selected 26 severe AH patients who had a favorable psychosocial profile and did not respond to standard treatment for early transplantation. This group of patients was compared to a matched group of 26 patients with severe AH who received standard treatment. The cumulative 6-mo survival rate of early transplant recipients was dramatically higher than controls (77% *vs* 23%, P < 0.001). The greatest benefits were observed within the first month after transplantation and were maintained during two years of follow-up (HR: 6.08; P = 0.004). Recurrence was reported in 12% of the cases. Singal *et al*[185] analyzed the UNOS database and identified 59 patients between 2004-2010 who underwent LT due to an AH diagnosis. The survival of grafts and AH transplant patients was compared with matched AC LT patients. Five-year graft and patient survival of AH and AC patients were 75% and 73% (P = 0.97) and 80% and 78% (P = 0.90), respectively. At New York's Mount Sinai Hospital, 94 patients with severe AH who did not respond to

medical therapy were evaluated for early LT. Overall, nine (9.6%) candidates with favorable psychosocial profiles underwent early LT, comprising 3% of all adult LT during the study period. The 6-mo survival rate was higher among those receiving early LT than matched controls (89% *vs* 11%, P < 0.001). Eight recipients were still alive at a median of 735 d, with one alcohol relapse[186]. Most recently, the John Hopkins Hospital group published their trial of early LT in severe AH[187]. Seventeen patients with severe AH who were transplanted early were compared with 26 AC patients with ≥ 6 mo of abstinence who were transplanted during the same period. The 6-mo survival was 100% and 89% for AH and alcoholic liver cirrhosis patients, respectively (P = 0.27). Alcohol relapse was similar in both groups: 23.5% and 29.2%, respectively (P > 0.99). Harmful drinking was higher among AH than cirrhotic patients, despite a lack of statistical significance (23.5% *vs* 11.5%, respectively; P = 0.42). A systematic review of 11 studies[188] concluded that survival and recurrence rates are similar in early-transplanted severe AH patients and AC transplant patients. Thus, despite the evidence that transplantation in patients with severe AH is feasible and presents good results in a well selected subgroup of patients, there is still a long way to go before considering this type of treatment as standard for this population[189,190].

POST-LT OUTCOMES IN ALD PATIENTS

Relapse

LT can cure liver disease, but not the underlying alcohol use disorder[135]. Therefore, the transplantation team should be alert to possible alcohol consumption relapse after LT. Although self-reported alcohol use is commonly of little value, biomarkers can be a helpful replacement. For instance, metabolites of alcohol, such as ethyl glucuronide, can reveal alcohol use up to 3-4 d after the last drink[191]. However, due to its high sensitivity, it can yield false-positive results when medications that contain alcohol or hand sanitizers that contain small amounts of ethanol are used[192]. Measuring ethyl glucuronide in hair samples can detect longer-term alcohol use[193].

A prospective study[146] following 208 ALD LT patients for up to 9 years found that 113 (54%) did not relapse. Among those who did (n = 95), four alcohol consumption patterns were identified: 1) the majority (n = 55; 28.6%) consumed small amounts infrequently; 2) others (n = 13; 6.4%) began by drinking moderate amounts early on, but reduced consumption over time; 3) some (n = 15; 7.9%) began drinking later and in increasing amounts; and 4) a minority (n = 12; 5.8%) resumed drinking shortly after LT and in increasing amounts. Patients in groups 2 and 4 (who started drinking early) were more likely to present with steatohepatitis (according to hepatic biopsy) and LT rejection, and all of those who died from ALD recurrence were in these groups. The researchers identified several pre-LT factors associated with relapse: An established diagnosis of alcohol dependence, a short sobriety period, a family history of alco-
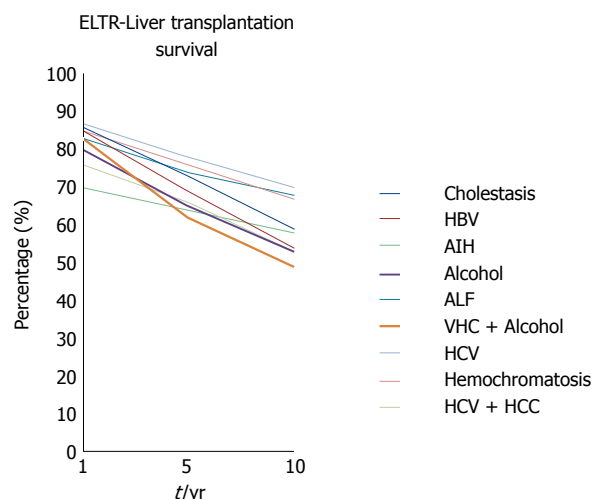
Figure 2 Survival of liver transplantation patients in Europe: 1, 5, and 10 years after liver transplantation. ELTR: European Liver Transplant Registry; HBV: Hepatitis B virus; AIH: Autoimmune hepatitis; ALF: Acute liver failure; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

holism, and the use of other substances[194].

A French multicenter retrospective study[147] analyzed the outcomes of 712 AC LT patients over approximately 9 years of follow up. At the end of the study, 128 patients (18%) had severe relapse (defined as a mean daily alcohol consumption > 20 g in women and > 30 g in men) for at least 6 mo. Of these, 41 (32%) developed AC on average 5 years after LT and 4 years after drinking again. A higher risk of relapse was observed in younger patients and those with a shorter sobriety period. Lastly, survival was lower in each time period in patients who developed recurrent AC compared to those who did not[195].

The results of these studies emphasize the importance of assessing alcohol use after LT and, if identified, taking measures to avoid the negative consequences.

Survival
Patient survival rates after LT for AC based on data from different parts of the world have been reported to be 81%-92%, 78%-86%, and 73%-86% at 1, 3, and 5 years, respectively[144]. There are slight differences with the survival rates of ALD LT patients: 93.1%, 87.4%, and 82% at 1, 3, and 5 years, respectively; these results were mostly obtained from small case series in localized geographic regions and regions with particular ethnic characteristics, such as Australia[196].

Survival rates after LT for ALD are comparable to those for patients who underwent LT for other causes[190]. The ELTR evaluated 121,546 patients who underwent LT in 1968-2015 in Europe, and the study found that the survival rates for AC patients (*n* = 22648) at 1, 5 and 10 years after LT were 86%, 73%, and 59%, respectively. These rates were greater than those observed for patients with HCV (80%, 65%, and 53%), HCC (83%, 62%, and 49%), acute liver failure (70%, 64%, and 58%), and hemochromatosis (76%, 66%, and 53%);

similar to those observed for patients with AC plus HCV or HBV (85%, 69%, and 54%); and a little lower than those observed for patients with cholestasis (87%, 78%, and 70%), autoimmune hepatitis (85%, 76%, and 67%), and HBV (83%, 74%, and 68%) (Figure 2)[197,198].

However, this somewhat equivalent survival among AC LT patients compared to other LT patient did not persist beyond 5 years due to the increased risk among AC LT patients of cardiorespiratory disease, cerebro-vascular events, and *de novo* malignancy. Although all LT recipients are at greater risk of *de novo* malignancy, the incidence rate is significantly higher in patients with ALD, particularly regarding oropharyngeal and lung cancers, which may be related to substance abuse and smoking history[197]. Nearly 40% of ALD LT recipients resume smoking soon after LT[26]. Therefore, pre- and post-LT follow-up efforts regarding ALD patients should be focused not only on alcohol consumption relapse, but also on treating and avoiding other modifiable risk factors such as tobacco smoking. Pre-LT psychiatric and psychosocial evaluation and post-LT follow-up with physicians, psychiatrists, and addiction specialists are important for dealing with these problems[26].

Recent studies have indicated that resumption of alcohol abuse following LT leads to significantly reduced survival rates. Patients who resumed heavy drinking have been reported to have 5- and 10-year survival rates of 69.5% and 20.1%, respectively, compared to 90.3% and 81.5%, respectively, in abstinent patients[7,190,199].

In Australia, 16% of patients who underwent LT for ALD fulfilled criteria for harmful relapse and 21% experienced any form of alcohol consumption relapse. Harmful relapse was associated with increased mortality. Based on a multivariate analysis, only two factors were independently associated with harmful relapse: Lack of prior participation in an alcohol rehabilitation program and single versus married status[196]. Younger women dependent on alcohol shortly before LT are at greatest risk of relapse[199].

Among patients who underwent LT for AC, there is improvement in the quality of life, mood, and cognitive functioning, with no difference compared to patients who underwent LT for non-AC etiologies. LT patients were able to return to society and lead active and prolific lives, irrespective of the indication for LT[190].

The key factor determining the outcome of LT for AC is intensive lifelong medical and psychological care. Post-LT surveillance might be much more important than pre-LT selection[37].

CONCLUSION

Alcohol is largely consumed worldwide, causing many diseases in several organs and systems. For patients with ALD, when end-stage liver disease is reached, the only chance of survival is LT. There are controversies and ethical dilemmas associated with the indication of LT for ALD. Accurate stratification of potential LT candidates should be performed to identify those most likely to

remain abstinent after LT. The survival of patients who underwent LT for AC is comparable to that of patients who underwent LT for other non-AC etiologies. Psychiatrists, psychologists, social workers and dependency specialists, along with the transplantation team, are essential in the post-LT follow-up of these patients. AC patients who underwent LT are most likely to develop cardiovascular illnesses and malignancies 5 years after LT; it is also imperative that these patients stop smoking.

The two most important words related to ALD are addiction and abstinence. The first represents hell in the life of the alcoholic and refers to the most difficult pathways of degradation and death. It can be circumvented in a complex and time-consuming process, often with relatively little success that, when obtained, should be preserved with the greatest possible effort of the patient and the surrounding supporters, in a constant struggle. The second represents redemption, achieved with much effort and persistence, and it must be preserved at all costs, constantly and permanently, always glimpsing the future of physical and emotional recovery. The sequelae may disappear and recovery, when possible, may be complete. Abstinence is the solid foundation on which recovery is based. However, it is fragile as well, and requires constant vigilance from the patient and the supporters, not to return to hell.

REFERENCES

1 **Busuttil RW**, DuBray BJ. Liver Transplantation for Alcoholic Hepatitis. *Ann Surg* 2017; **265**: 30-31 [PMID: 27611611 DOI: 10.1097/SLA.0000000000001994]

2 **Kim WR**, Lake JR, Smith JM, Skeans MA, Schladt DP, Edwards EB, Harper AM, Wainright JL, Snyder JJ, Israni AK, Kasiske BL. Liver. *Am J Transplant* 2016; **16** Suppl 2: 69-98 [PMID: 26755264 DOI: 10.1111/ajt.13668]

3 **Lumeng L**, Crabb DW. Genetic aspects and risk factors in alcoholism and alcoholic liver disease. *Gastroenterology* 1994; **107**: 572-578 [PMID: 8039633 DOI: 10.1016/0016-5085(94)90185-6]

4 **Rehm J**, Mathers C, Popova S, Thavorncharoensap M, Teerawattananon Y, Patra J. Global burden of disease and injury and economic cost attributable to alcohol use and alcohol-use disorders. *Lancet* 2009; **373**: 2223-2233 [PMID: 19560604 DOI: 10.1016/S0140-6736(09)60746-7]

5 **Marroni CA,** Bona S, Fleck Junior AM, Moreira AJ, Mariante Neto G, Rodrigues G, Marroni CP, Coral GP, Ayres R, Schneider ACR, da Silveira TR, Brandão ABM, Marroni NP. Clinical and experimental alcoholic liver disease. *J Liver Clin Res* 2016; **3**: 1028

6 **Lucey MR**. Liver transplantation in the alcoholic patient. In: Maddrey WC, Schiff ER, Sorell MF, editors. Transplantation of the liver. Philadelphia: Lippincott Williams Wilkins, 2001: 319-326

7 **Varma V**, Webb K, Mirza DF. Liver transplantation for alcoholic liver disease. *World J Gastroenterol* 2010; **16**: 4377-4393 [PMID: 20845504 DOI: 10.3748/wjg.v16.i35.4377]

8 **Scharschmidt BF**. Human liver transplantation: analysis of data on 540 patients from four centers. *Hepatology* 1984; **4**: 95S-101S [PMID: 6363266 DOI: 10.1002/hep.1840040723]

9 **MacDougall BR**, Williams R. Indication and assessment for orthotopic liver transplantation. In: Calne RY. Liver transplantation. London: Grune Stratton, 1983: 59

10 **Neuberger J**, Williams R. Indication and assessment for Liver Grafting. In: Calne R Y. Liver Transplantation. 2nd ed. London: Grune Stratton, 1987: 63

11 **Starzl TE**, Van Thiel D, Tzakis AG, Iwatsuki S, Todo S, Marsh JW, Koneru B, Staschak S, Stieber A, Gordon RD. Orthotopic liver transplantation for alcoholic cirrhosis. *JAMA* 1988; **260**: 2542-2544 [PMID: 3050180 DOI: 10.1001/jama.1988.03410170090040]

12 **Beresford TP**, Turcotte JG, Merion R, Burtch G, Blow FC, Campbell D, Brower KJ, Coffman K, Lucey M. A rational approach to liver transplantation for the alcoholic patient. *Psychosomatics* 1990; **31**: 241-254 [PMID: 2095755 DOI: 10.1016/S0033-3182(90)72160-3]

13 **Lucey MR**, Merion RM, Henley KS, Campbell DA Jr, Turcotte JG, Nostrant TT, Blow FC, Beresford TP. Selection for and outcome of liver transplantation in alcoholic liver disease. *Gastroenterology* 1992; **102**: 1736-1741 [PMID: 1568583 DOI: 10.1016/0016-5085(92)91737-O]

14 **Bird GL**, Williams R. Treatment of advanced alcoholic liver disease. *Alcohol Alcohol* 1990; **25**: 197-206 [PMID: 2198035 DOI: 10.1093/oxfordjournals.alcalc.a044993]

15 **Bird GL**, O'Grady JG, Harvey FA, Calne RY, Williams R. Liver transplantation in patients with alcoholic cirrhosis: selection criteria and rates of survival and relapse. *BMJ* 1990; **301**: 15-17 [PMID: 2383700 DOI: 10.1136/bmj.301.6742.15]

16 **Kumar S**, Stauber RE, Gavaler JS, Basista MH, Dindzans VJ, Schade RR, Rabinovitz M, Tarter RE, Gordon R, Starzl TE. Orthotopic liver transplantation for alcoholic liver disease. *Hepatology* 1990; **11**: 159-164 [PMID: 2307394 DOI: 10.1002/hep.1840110202]

17 **McCurry KR**, Baliga P, Merion RM, Ham JM, Lucey MR, Beresford TP, Turcotte JG, Campbell DA Jr. Resource utilization and outcome of liver transplantation for alcoholic cirrhosis. A case-control study. *Arch Surg* 1992; **127**: 772-776; discussion 776-777 [PMID: 1524475 DOI: 10.1001/archsurg.1992.01420070024007]

18 **Poynard T**, Barthelemy P, Fratte S, Boudjema K, Doffoel M, Vanlemmens C, Miguet JP, Mantion G, Messner M, Launois B. Evaluation of efficacy of liver transplantation in alcoholic cirrhosis by a case-control study and simulated controls. *Lancet* 1994; **344**: 502-507 [PMID: 7914613 DOI: 10.1016/S0140-6736(94)91897-X]

19 European Liver Transplant Registry (ELTR). [internet] [cited 16 May 2018]. Available from: http://www.eltr.org/Evolution-of-LTs-in-Europe.html

20 **Kotlyar DS**, Burke A, Campbell MS, Weinrieb RM. A critical review of candidacy for orthotopic liver transplantation in alcoholic liver disease. *Am J Gastroenterol* 2008; **103**: 734-43; quiz 744 [PMID: 18081918 DOI: 10.1111/j.1572-0241.2007.01691.x]

21 **Marroni CA**. Management of alcohol recurrence before and after liver transplantation. *Clin Res Hepatol Gastroenterol* 2015; **39** Suppl 1: S109-S114 [PMID: 26193869 DOI: 10.1016/j.clinre.2015.06.005]

22 **Weinrieb RM**, Van Horn DH, McLellan AT, Lucey MR. Interpreting the significance of drinking by alcohol-dependent liver transplant patients: fostering candor is the key to recovery. *Liver Transpl* 2000; **6**: 769-776 [PMID: 11084066 DOI: 10.1053/jlts.2000.18497]

23 **Tandon P**, Goodman KJ, Ma MM, Wong WW, Mason AL, Meeberg G, Bergsten D, Carbonneau M, Bain VG. A shorter duration of pre-transplant abstinence predicts problem drinking after liver transplantation. *Am J Gastroenterol* 2009; **104**: 1700-1706 [PMID: 19471253 DOI: 10.1038/ajg.2009.226]

24 **Burra P**, Lucey MR. Liver transplantation in alcoholic patients. *Transpl Int* 2005; **18**: 491-498 [PMID: 15819795 DOI: 10.1111/j.1432-2277.2005.00079.x]

25 NHS Organ Donation, Liver advisory group alcohol guidelines. 2017. Cited 2018-05-16 Available from: URL: http://odt.nhs.uk/pdf/liver_selection_policy.pdf

26 **Iruzubieta P**, Crespo J, Fábrega E. Long-term survival after liver transplantation for alcoholic liver disease. *World J Gastroenterol* 2013; **19**: 9198-9208 [PMID: 24409048 DOI: 10.3748/wjg.v19.i48.9198]

27 **Vaillant GE**. A 60-year follow-up of alcoholic men. *Addiction* 2003; **98**: 1043-1051 [PMID: 12873238 DOI: 10.1046/j.1360-0443.2003.00422.x]

28 **Rice JP**, Eickhoff J, Agni R, Ghufran A, Brahmbhatt R, Lucey MR. Abusive drinking after liver transplantation is associated with allograft loss and advanced allograft fibrosis. *Liver Transpl* 2013; **19**: 1377-1386 [PMID: 24115392 DOI: 10.1002/lt.23762]

29   **Testino G**, Burra P, Bonino F, Piani F, Sumberaz A, Peressutti R, Giannelli Castiglione A, Patussi V, Fanucchi T, Ancarani O, De Cerce G, Iannini AT, Greco G, Mosti A, Durante M, Babocci P, Quartini M, Mioni D, Aricò S, Baselice A, Leone S, Lozer F, Scafato E, Borro P; Group of Italian Regions. Acute alcoholic hepatitis, end stage alcoholic liver disease and liver transplantation: an Italian position statement. *World J Gastroenterol* 2014; **20**: 14642-14651 [PMID: 25356027 DOI: 10.3748/wjg.v20.i40.14642]

30   **Dutkowski P**, Linecker M, DeOliveira ML, Müllhaupt B, Clavien PA. Challenges to liver transplantation and strategies to improve outcomes. *Gastroenterology* 2015; **148**: 307-323 [PMID: 25224524 DOI: 10.1053/j.gastro.2014.08.045]

31   **Rustad JK**, Stern TA, Prabhakar M, Musselman D. Risk factors for alcohol relapse following orthotopic liver transplantation: a systematic review. *Psychosomatics* 2015; **56**: 21-35 [PMID: 25619671 DOI: 10.1016/j.psym.2014.09.006]

32   **Gramenzi A**, Biselli M, Andreone P. Authors' reply: comment to "liver transplantation for patients with alcoholic liver disease: an open question". *Dig Liver Dis* 2013; **45**: 81 [PMID: 22898145 DOI: 10.1016/j.dld.2012.07.006]

33   **Leong J**, Im GY. Evaluation and selection of the patient with alcoholic liver disease for liver transplant. *Clin Liver Dis* 2012; **16**: 851-863 [PMID: 23101986 DOI: 10.1016/j.cld.2012.08.012]

34   United Network for Organ Sharing (UNOS). [cited 16 May 2018] In: Minimal criteria for liver transplantation. [internet] 2003. Available from: https://unos.org/wp-content/uploads/unos/Liver_patient.pdf

35   **European Association for the Study of Liver**. EASL clinical practical guidelines: management of alcoholic liver disease. *J Hepatol* 2012; **57**: 399-420 [PMID: 22633836 DOI: 10.1016/j.jhep.2012.04.004]

36   **Testino G**, Leone S, Sumberaz A, Borro P. Liver transplantation in alcoholic patients. *Alcohol Clin Exp Res* 2014; **38**: 1800-1802 [PMID: 24033401 DOI: 10.1111/acer.12242]

37   **Berlakovich GA**. Challenges in transplantation for alcoholic liver disease. *World J Gastroenterol* 2014; **20**: 8033-8039 [PMID: 25009374 DOI: 10.3748/wjg.v20.i25.8033]

38   **Aravinthan AD**, Barbas AS, Doyle AC, Tazari M, Sapisochin G, Cattral MS, Ghanekar A, McGilvray ID, Selzner M, Greig PD, Bhat M, Selzner N, Grant DR, Lilly LB, Renner EL. Characteristics of liver transplant candidates delisted following recompensation and predictors of such delisting in alcohol-related liver disease: a case-control study. *Transpl Int* 2017; **30**: 1140-1149 [PMID: 28686307 DOI: 10.1111/tri.13008]

39   **Hajifathalian K**, Humberson A, Hanouneh MA, Barnes DS, Arora Z, Zein NN, Eghtesad B, Kelly D, Hanouneh IA. Ohio solid organ transplantation consortium criteria for liver transplantation in patients with alcoholic liver disease. *World J Hepatol* 2016; **8**: 1149-1154 [PMID: 27721920 DOI: 10.4254/wjh.v8.i27.1149]

40   **Russ KB**, Chen NW, Kamath PS, Shah VH, Kuo YF, Singal AK. Alcohol Use after Liver Transplantation is Independent of Liver Disease Etiology. *Alcohol Alcohol* 2016; **51**: 698-701 [PMID: 27267907 DOI: 10.1093/alcalc/agw032]

41   **Testino G**, Leone S. Alcohol and Liver Transplantation. *Alcohol Alcohol* 2017; **52**: 126 [PMID: 27600939 DOI: 10.1093/alcalc/agw056]

42   **Hejlova I**, Honsova E, Sticova E, Lanska V, Hucl T, Spicak J, Jirsa M, Trunecka P. Prevalence and risk factors of steatosis after liver transplantation and patient outcomes. *Liver Transpl* 2016; **22**: 644-655 [PMID: 26707008 DOI: 10.1002/lt.24393]

43   **Mukthinuthalapati PK**, Gotur R, Ghabril M. Incidence, risk factors and outcomes of de novo malignancies post liver transplantation. *World J Hepatol* 2016; **8**: 533-544 [PMID: 27134701 DOI: 10.4254/wjh.v8.i12.533]

44   **Obed A**, Stern S, Jarrad A, Lorf T. Six month abstinence rule for liver transplantation in severe alcoholic liver disease patients. *World J Gastroenterol* 2015; **21**: 4423-4426 [PMID: 25892898 DOI: 10.3748/wjg.v21.i14.4423]

45   **Burroughs AK**. Liver transplantation for severe alcoholic hepatitis saves lives. *J Hepatol* 2012; **57**: 451-452 [PMID: 22285999 DOI: 10.1016/j.jhep.2012.01.003]

46   **Duvoux C**. [Liver transplantation: which indications? which results?]. *Presse Med* 2001; **30**: 711-716 [PMID: 11360736]

47   **Neuberger J**. Public and professional attitudes to transplanting alcoholic patients. *Liver Transpl* 2007; **13**: S65-S68 [PMID: 17969090 DOI: 10.1002/lt.21337]

48   **Yates WR**, Labrecque DR, Pfab D. The reliability of alcoholism history in patients with alcohol-related cirrhosis. *Alcohol Alcohol* 1998; **33**: 488-494 [PMID: 9811201 DOI: 10.1093/alcalc/33.5.488]

49   **Neuberger J**, Adams D, MacMaster P, Maidment A, Speed M. Assessing priorities for allocation of donor liver grafts: survey of public and clinicians. *BMJ* 1998; **317**: 172-175 [PMID: 9665895 DOI: 10.1136/bmj.317.7152.172]

50   **Ferraguti G**, Pascale E, Lucarelli M. Alcohol addiction: a molecular biology perspective. *Curr Med Chem* 2015; **22**: 670-684 [PMID: 25544474 DOI: 10.2174/0929867321666141229103158]

51   **Neuberger J**, Lucey MR. Liver transplantation: Practice and management. London: BMJ Publishing Group, 1994: 400

52   **Gallegos-Orozco JF**, Charlton MR. Alcoholic Liver Disease and Liver Transplantation. *Clin Liver Dis* 2016; **20**: 521-534 [PMID: 27373614 DOI: 10.1016/j.cld.2016.02.009]

53   **Burra P**, Senzolo M, Adam R, Delvart V, Karam V, Germani G, Neuberger J; ELITA; ELTR Liver Transplant Centers. Liver transplantation for alcoholic liver disease in Europe: a study from the ELTR (European Liver Transplant Registry). *Am J Transplant* 2010; **10**: 138-148 [PMID: 19951276 DOI: 10.1111/j.1600-6143.2009.02869.x]

54   **Singal AK**, Guturu P, Hmoud B, Kuo YF, Salameh H, Wiesner RH. Evolving frequency and outcomes of liver transplantation based on etiology of liver disease. *Transplantation* 2013; **95**: 755-760 [PMID: 23370710 DOI: 10.1097/TP.0b013e31827afb3a]

55   **Adam R**, Karam V, Delvart V, O'Grady J, Mirza D, Klempnauer J, Castaing D, Neuhaus P, Jamieson N, Salizzoni M, Pollard S, Lerut J, Paul A, Garcia-Valdecasas JC, Rodríguez FS, Burroughs A; All contributing centers (www.eltr.org); European Liver and Intestine Transplant Association (ELITA). Evolution of indications and results of liver transplantation in Europe. A report from the European Liver Transplant Registry (ELTR). *J Hepatol* 2012; **57**: 675-688 [PMID: 22609307 DOI: 10.1016/j.jhep.2012.04.015]

56   **Goldberg D**, Ditah IC, Saeian K, Lalehzari M, Aronsohn A, Gorospe EC, Charlton M. Changes in the Prevalence of Hepatitis C Virus Infection, Nonalcoholic Steatohepatitis, and Alcoholic Liver Disease Among Patients With Cirrhosis or Liver Failure on the Waitlist for Liver Transplantation. *Gastroenterology* 2017; **152**: 1090-1099.e1 [PMID: 28088461 DOI: 10.1053/j.gastro.2017.01.003]

57   **Kim WR**, Lake JR, Smith JM, Schladt DP, Skeans MA, Harper AM, Wainright JL, Snyder JJ, Israni AK, Kasiske BL. OPTN/SRTR 2016 Annual Data Report: Liver. *Am J Transplant* 2018; **18** Suppl 1: 172-253 [PMID: 29292603 DOI: 10.1111/ajt.14559]

58   **Gaglio PJ Jr**, Gaglio PJ Sr. Complications in patients with alcohol-associated liver disease who undergo liver transplantation. *Clin Liver Dis* 2012; **16**: 865-875 [PMID: 23101987 DOI: 10.1016/j.cld.2012.08.013]

59   **Singal AK**, Charlton MR. Nutrition in alcoholic liver disease. *Clin Liver Dis* 2012; **16**: 805-826 [PMID: 23101983 DOI: 10.1016/j.cld.2012.08.009]

60   **Addolorato G**, Bataller R, Burra P, DiMartini A, Graziadei I, Lucey MR, Mathurin P, O'Grady J, Pageaux G, Berenguer M. Liver Transplantation for Alcoholic Liver Disease. *Transplantation* 2016; **100**: 981-987 [PMID: 26985744 DOI: 10.1097/TP.0000000000001156]

61   **Jepsen P**, Lash TL, Vilstrup H. The clinical course of alcoholic cirrhosis: development of comorbid diseases. A Danish nationwide cohort study. *Liver Int* 2016; **36**: 1696-1703 [PMID: 27124269 DOI: 10.1111/liv.13151]

62   **Mazurak VC**, Tandon P, Montano-Loza AJ. Nutrition and the transplant candidate. *Liver Transpl* 2017; **23**: 1451-1464 [PMID: 29072825 DOI: 10.1002/lt.24848]

63   **Caly WR**, Strauss E, Carrilho FJ, Laudanna AA. Different degrees of malnutrition and immunological alterations according to the aetiology of cirrhosis: a prospective and sequential study. *Nutr J*

2003; **2**: 10 [PMID: 14613508 DOI: 10.1186/1475-2891-2-10]

64   **Purnak T**, Yilmaz Y. Liver disease and malnutrition. *Best Pract Res Clin Gastroenterol* 2013; **27**: 619-629 [PMID: 24090946 DOI: 10.1016/j.bpg.2013.06.018]

65   **González-Reimers E**, Santolaria-Fernández F, Martín-González MC, Fernández-Rodríguez CM, Quintero-Platt G. Alcoholism: a systemic proinflammatory condition. *World J Gastroenterol* 2014; **20**: 14660-14671 [PMID: 25356029 DOI: 10.3748/wjg.v20.i40.14660]

66   **Kasper DL**, Fauci AS, Hauser SL, Longo DL, Jameson JL, Loscalzo J. Harrisson´s manual of medicine. 19th ed. New York: McGraw Hill, 2016

67   **Rocco A**, Compare D, Angrisani D, Sanduzzi Zamparelli M, Nardone G. Alcoholic disease: liver and beyond. *World J Gastroenterol* 2014; **20**: 14652-14659 [PMID: 25356028 DOI: 10.3748/wjg.v20.i40.14652]

68   **Kortas DY**, Haas LS, Simpson WG, Nickl NJ 3rd, Gates LK Jr. Mallory-Weiss tear: predisposing factors and predictors of a complicated course. *Am J Gastroenterol* 2001; **96**: 2863-2865 [PMID: 11693318 DOI: 10.1111/j.1572-0241.2001.04239.x]

69   **Szabo G**, Mandrekar P, Oak S, Mayerle J. Effect of ethanol on inflammatory responses. Implications for pancreatitis. *Pancreatology* 2007; **7**: 115-123 [PMID: 17592223 DOI: 10.1159/000104236]

70   **Donnadieu-Rigole H**, Mura T, Portales P, Duroux-Richard I, Bouthier M, Eliaou JF, Perney P, Apparailly F. Effects of alcohol withdrawal on monocyte subset defects in chronic alcohol users. *J Leukoc Biol* 2016; **100**: 1191-1199 [PMID: 27256567 DOI: 10.1189/jlb.5A0216-060RR]

71   **Hassan AN**. Patients With Alcohol Use Disorder Co-Occurring With Depression and Anxiety Symptoms: Diagnostic and Treatment Initiation Recommendations. *J Clin Psychiatry* 2018; **79**: [PMID: 29244266 DOI: 10.4088/JCP.17ac11999]

72   **Burra P**, Zanetto A, Germani G. Liver Transplantation for Alcoholic Liver Disease and Hepatocellular Carcinoma. *Cancers* (Basel) 2018; **10**: [PMID: 29425151 DOI: 10.3390/cancers10020046]

73   **El-Serag HB**, Mason AC. Risk factors for the rising rates of primary liver cancer in the United States. *Arch Intern Med* 2000; **160**: 3227-3230 [PMID: 11088082 DOI: 10.1001/archinte.160.21.3227]

74   **Hassan MM**, Hwang LY, Hatten CJ, Swaim M, Li D, Abbruzzese JL, Beasley P, Patt YZ. Risk factors for hepatocellular carcinoma: synergism of alcohol with viral hepatitis and diabetes mellitus. *Hepatology* 2002; **36**: 1206-1213 [PMID: 12395331 DOI: 10.1053/jhep.2002.36780]

75   **Marot A**, Henrion J, Knebel JF, Moreno C, Deltenre P. Alcoholic liver disease confers a worse prognosis than HCV infection and non-alcoholic fatty liver disease among patients with cirrhosis: An observational study. *PLoS One* 2017; **12**: e0186715 [PMID: 29077714 DOI: 10.1371/journal.pone.0186715]

76   **Bucci L**, Garuti F, Camelli V, Lenzi B, Farinati F, Giannini EG, Ciccarese F, Piscaglia F, Rapaccini GL, Di Marco M, Caturelli E, Zoli M, Borzio F, Sacco R, Maida M, Felder M, Morisco F, Gasbarrini A, Gemini S, Foschi FG, Missale G, Masotto A, Affronti A, Bernardi M, Trevisani F; Italian Liver Cancer (ITA.LI.CA) Group; Italian Liver Cancer ITA LI CA Group. Comparison between alcohol- and hepatitis C virus-related hepatocellular carcinoma: clinical presentation, treatment and outcome. *Aliment Pharmacol Ther* 2016; **43**: 385-399 [PMID: 26662476 DOI: 10.1111/apt.13485]

77   **West J**, Card TR, Aithal GP, Fleming KM. Risk of hepatocellular carcinoma among individuals with different aetiologies of cirrhosis: a population-based cohort study. *Aliment Pharmacol Ther* 2017; **45**: 983-990 [PMID: 28144999 DOI: 10.1111/apt.13961]

78   **European Association For The Study Of The Liver.**; European Organisation For Research And Treatment Of Cancer. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. *J Hepatol* 2012; **56**: 908-943 [PMID: 22424438 DOI: 10.1016/j.jhep.2011.12.001]

79   **Mancebo A**, González-Diéguez ML, Cadahía V, Varela M, Pérez R, Navascués CA, Sotorríos NG, Martínez M, Rodrigo L, Rodríguez M. Annual incidence of hepatocellular carcinoma among patients with alcoholic cirrhosis and identification of risk groups. *Clin Gastroenterol Hepatol* 2013; **11**: 95-101 [PMID: 22982095 DOI: 10.1016/j.cgh.2012.09.007]

80   **Dasarathy S**. Consilience in sarcopenia of cirrhosis. *J Cachexia Sarcopenia Muscle* 2012; **3**: 225-237 [PMID: 22648736 DOI: 10.1007/s13539-012-0069-3]

81   **Merli M**, Romiti A, Riggio O, Capocaccia L. Optimal nutritional indexes in chronic liver disease. *JPEN J Parenter Enteral Nutr* 1987; **11**: 130S-134S [PMID: 3669265 DOI: 10.1177/014860718701100521]

82   **Romiti A**, Merli M, Martorano M, Parrilli G, Martino F, Riggio O, Truscelli A, Capocaccia L, Budillon G. Malabsorption and nutritional abnormalities in patients with liver cirrhosis. *Ital J Gastroenterol* 1990; **22**: 118-123 [PMID: 2131941]

83   **Guglielmi FW**, Panella C, Buda A, Budillon G, Caregaro L, Clerici C, Conte D, Federico A, Gasbarrini G, Guglielmi A, Loguercio C, Losco A, Martines D, Mazzuoli S, Merli M, Mingrone G, Morelli A, Nardone G, Zoli G, Francavilla A. Nutritional state and energy balance in cirrhotic patients with or without hypermetabolism. Multicentre prospective study by the 'Nutritional Problems in Gastroenterology' Section of the Italian Society of Gastroenterology (SIGE). *Dig Liver Dis* 2005; **37**: 681-688 [PMID: 15978878 DOI: 10.1016/j.dld.2005.03.010]

84   **Dasarathy S**. Nutrition and Alcoholic Liver Disease: Effects of Alcoholism on Nutrition, Effects of Nutrition on Alcoholic Liver Disease, and Nutritional Therapies for Alcoholic Liver Disease. *Clin Liver Dis* 2016; **20**: 535-550 [PMID: 27373615 DOI: 10.1016/j.cld.2016.02.010]

85   **McClain CJ**, Barve SS, Barve A, Marsano L. Alcoholic liver disease and malnutrition. *Alcohol Clin Exp Res* 2011; **35**: 815-820 [PMID: 21284673 DOI: 10.1111/j.1530-0277.2010.01405.x]

86   **Hara N**, Iwasa M, Sugimoto R, Mifuji-Moroka R, Yoshikawa K, Terasaka E, Hattori A, Ishidome M, Kobayashi Y, Hasegawa H, Iwata K, Takei Y. Sarcopenia and Sarcopenic Obesity Are Prognostic Factors for Overall Survival in Patients with Cirrhosis. *Intern Med* 2016; **55**: 863-870 [PMID: 27086797 DOI: 10.2169/internalmedicine.55.5676]

87   **Tsien C**, Garber A, Narayanan A, Shah SN, Barnes D, Eghtesad B, Fung J, McCullough AJ, Dasarathy S. Post-liver transplantation sarcopenia in cirrhosis: a prospective evaluation. *J Gastroenterol Hepatol* 2014; **29**: 1250-1257 [PMID: 24443785 DOI: 10.1111/jgh.12524]

88   **Dasarathy S**. Posttransplant sarcopenia: an underrecognized early consequence of liver transplantation. *Dig Dis Sci* 2013; **58**: 3103-3111 [PMID: 23912247 DOI: 10.1007/s10620-013-2791-x]

89   **Tsien C**, Shah SN, McCullough AJ, Dasarathy S. Reversal of sarcopenia predicts survival after a transjugular intrahepatic portosystemic stent. *Eur J Gastroenterol Hepatol* 2013; **25**: 85-93 [PMID: 23011041 DOI: 10.1097/MEG.0b013e328359a759]

90   **Fernandes SA**, Bassani L, Nunes FF, Aydos ME, Alves AV, Marroni CA. Nutritional assessment in patients with cirrhosis. *Arq Gastroenterol* 2012; **49**: 19-27 [PMID: 22481682 DOI: 10.1590/S0004-28032012000100005]

91   **Gonzalez MC**, Correia MITD, Heymsfield SB. A requiem for BMI in the clinical setting. *Curr Opin Clin Nutr Metab Care* 2017; **20**: 314-321 [PMID: 28768291 DOI: 10.1097/MCO.0000000000000395]

92   **Figueiredo FA**, Perez RM, Freitas MM, Kondo M. Comparison of three methods of nutritional assessment in liver cirrhosis: subjective global assessment, traditional nutritional parameters, and body composition analysis. *J Gastroenterol* 2006; **41**: 476-482 [PMID: 16799890 DOI: 10.1007/s00535-006-1794-1]

93   **Gottschall CB**, Alvares-da-Silva MR, Camargo AC, Burtett RM, da Silveira TR. [Nutritional assessment in patients with cirrhosis: the use of indirect calorimetry]. *Arq Gastroenterol* 2004; **41**: 220-224 [PMID: 15806264 DOI: 10.1590/S0004-28032004000400004]

94   **Hasse J**, Strong S, Gorman MA, Liepa G. Subjective global assessment: alternative nutrition-assessment technique for liver-transplant candidates. *Nutrition* 1993; **9**: 339-343 [PMID: 8400590]

95   Nutritional status in cirrhosis. Italian Multicentre Cooperative Project on Nutrition in Liver Cirrhosis. *J Hepatol* 1994; **21**: 317-325 [PMID: 7836699]

96   **Naveau S**, Belda E, Borotto E, Genuist F, Chaput JC. Comparison of clinical judgment and anthropometric parameters for evaluating nutritional status in patients with alcoholic liver disease. *J Hepatol* 1995; **23**: 234-235 [PMID: 7499801 DOI: 10.1016/0168-8278(95)80344-0]

97   **Bauer J**, Capra S, Ferguson M. Use of the scored Patient-Generated Subjective Global Assessment (PG-SGA) as a nutrition assessment tool in patients with cancer. *Eur J Clin Nutr* 2002; **56**: 779-785 [PMID: 12122555 DOI: 10.1038/sj.ejcn.1601412]

98   **Fernandes SA**, Gonzalez MC, Bassani L, Miranda D, Pivatto B, Harter DL, Marroni CA. Is the phase angle, a prognostic indicator for nutritional status in cirrhotic patients? *J Antivir Antiretrovir* 2013; **S3**: 004 [DOI: 10.4172/jaa.S3-004]

99   **Álvares-Da-Siva MR**, Silveira TR. Hand-grip strength or muscle mass in cirrhotic patients: who is the best? *Nutrition* 2006; **22**: 218-219 [DOI: 10.1016/j.nut.2005.06.001]

100 **Lee SY**, Gallagher D. Assessment methods in human body composition. *Curr Opin Clin Nutr Metab Care* 2008; **11**: 566-572 [PMID: 18685451 DOI: 10.1097/MCO.0b013e32830b5f23]

101 **Lehnert ME**, Clarke DD, Gibbons JG, Ward LC, Golding SM, Shepherd RW, Cornish BH, Crawford DH. Estimation of body water compartments in cirrhosis by multiple-frequency bioelectrical-impedance analysis. *Nutrition* 2001; **17**: 31-34 [PMID: 11165885 DOI: 10.1016/S0899-9007(00)00473-1]

102 **Dasarathy J**, Alkhouri N, Dasarathy S. Changes in body composition after transjugular intrahepatic portosystemic stent in cirrhosis: a critical review of literature. *Liver Int* 2011; **31**: 1250-1258 [PMID: 21745273 DOI: 10.1111/j.1478-3231.2011.02498.x]

103 **Shen W**, Punyanitya M, Wang Z, Gallagher D, St-Onge MP, Albu J, Heymsfield SB, Heshka S. Total body skeletal muscle and adipose tissue volumes: estimation from a single abdominal cross-sectional image. *J Appl Physiol (1985)* 2004; **97**: 2333-2338 [PMID: 15310748 DOI: 10.1152/japplphysiol.00744.2004]

104 **Bemben MG**. Use of diagnostic ultrasound for assessing muscle size. *J Strength Cond Res* 2002; **16**: 103-108 [PMID: 11834114]

105 **Glass C**, Hipskind P, Tsien C, Malin SK, Kasumov T, Shah SN, Kirwan JP, Dasarathy S. Sarcopenia and a physiologically low respiratory quotient in patients with cirrhosis: a prospective controlled study. *J Appl Physiol (1985)* 2013; **114**: 559-565 [PMID: 23288550 DOI: 10.1152/japplphysiol.01042.2012]

106 **Ellis KJ**. Human body composition: in vivo methods. *Physiol Rev* 2000; **80**: 649-680 [PMID: 10747204 DOI: 10.1152/physrev.2000.80.2.649]

107 **Marroni CA**, Miranda D, Boemeke L, Fernandes SA. Phase Angle Bioelectrical Impedance Analysis (BIA) as a Biomarker Tool for Liver Disease. In: Patel VB Preedy VR. Biomarkers in Liver Disease (Biomarkers in Disease: Methods, Discoveries and Applications). Springer Science, 2017: 735-751

108 **Thapaliya S**, Runkana A, McMullen MR, Nagy LE, McDonald C, Naga Prasad SV, Dasarathy S. Alcohol-induced autophagy contributes to loss in skeletal muscle mass. *Autophagy* 2014; **10**: 677-690 [PMID: 24492484 DOI: 10.4161/auto.27918]

109 **Sinclair M**, Grossmann M, Hoermann R, Angus PW, Gow PJ. Testosterone therapy increases muscle mass in men with cirrhosis and low testosterone: A randomised controlled trial. *J Hepatol* 2016; **65**: 906-913 [PMID: 27312945 DOI: 10.1016/j.jhep.2016.06.007]

110 **Rennie MJ**. Anabolic resistance: the effects of aging, sexual dimorphism, and immobilization on human muscle protein turnover. *Appl Physiol Nutr Metab* 2009; **34**: 377-381 [PMID: 19448702 DOI: 10.1139/H09-012]

111 **Qiu J**, Thapaliya S, Runkana A, Yang Y, Tsien C, Mohan ML, Narayanan A, Eghtesad B, Mozdziak PE, McDonald C, Stark GR, Welle S, Naga Prasad SV, Dasarathy S. Hyperammonemia in cirrhosis induces transcriptional regulation of myostatin by an NF-κB-mediated mechanism. *Proc Natl Acad Sci USA* 2013; **110**: 18162-18167 [PMID: 24145431 DOI: 10.1073/pnas.1317049110]

112 **Qiu J**, Tsien C, Thapalaya S, Narayanan A, Weihl CC, Ching JK, Eghtesad B, Singh K, Fu X, Dubyak G, McDonald C, Almasan A, Hazen SL, Naga Prasad SV, Dasarathy S. Hyperammonemia-mediated autophagy in skeletal muscle contributes to sarcopenia of cirrhosis. *Am J Physiol Endocrinol Metab* 2012; **303**: E983-E993 [PMID: 22895779 DOI: 10.1152/ajpendo.00183.2012]

113 **Deutrich Aydos ME**, Alves Fernandes S, Feijó Nunes F, Bassani L, Rigon Leonhardt L, Lazzarotto Harter D, Pivato B, Miranda D, Augusto Marroni C. Seguimiento a un año del estado nutricional de los pacientes sometidos a trasplante hepático. *Nutr Hosp* 2016; **33**: 14-20 [PMID: 27019235 DOI: 10.20960/nh.8]

114 **Selberg O**, Selberg D. Norms and correlates of bioimpedance phase angle in healthy human subjects, hospitalized patients, and patients with liver cirrhosis. *Eur J Appl Physiol* 2002; **86**: 509-516 [PMID: 11944099 DOI: 10.1007/s00421-001-0570-4]

115 **Ruiz-Margáin A**, Macías-Rodríguez RU, Duarte-Rojo A, Ríos-Torres SL, Espinosa-Cuevas Á, Torre A. Malnutrition assessed through phase angle and its relation to prognosis in patients with compensated liver cirrhosis: a prospective cohort study. *Dig Liver Dis* 2015; **47**: 309-314 [PMID: 25618555 DOI: 10.1016/j.dld.2014.12.015]

116 **Baumgartner RN**, Chumlea WC, Roche AF. Bioelectric impedance phase angle and body composition. *Am J Clin Nutr* 1988; **48**: 16-23 [PMID: 3389323 DOI: 10.1093/ajcn/48.1.16]

117 **Fernandes SA**, Bona S, Cerski CT, Marroni NP, Marroni CA. ALTERATION OF TASTE BUDS IN EXPERIMENTAL CIRRHOSIS. Is there correlation with human hypogeusia? *Arq Gastroenterol* 2016; **53**: 278-284 [PMID: 27706460 DOI: 10.1590/S0004-28032016000400013]

118 **Dorna Mde S**, Santos LA, Gondo FF, Augusti L, de Campos Franzoni L, Sassaki LY, Romeiro FG, de Paiva SA, Minicucci MF, Silva GF. Phase angle is associated with advanced fibrosis in patients chronically infected with hepatitis C virus. *Life Sci* 2016; **154**: 30-33 [PMID: 26896689 DOI: 10.1016/j.lfs.2016.02.061]

119 **Kahraman A**, Hilsenbeck J, Nyga M, Ertle J, Wree A, Plauth M, Gerken G, Canbay AE. Bioelectrical impedance analysis in clinical practice: implications for hepatitis C therapy BIA and hepatitis C. *Virol J* 2010; **7**: 191 [PMID: 20712878 DOI: 10.1186/1743-422X-7-191]

120 **Piccoli A**, Rossi B, Pillon L, Bucciante G. A new method for monitoring body fluid variation by bioimpedance analysis: the RXc graph. *Kidney Int* 1994; **46**: 534-539 [PMID: 7967368 DOI: 10.1038/ki.1994.305]

121 **Piccoli A**, Pastori G. BIVA Software. Padova, Italy: Department of Medical and Surgical Sciences, University of Padova, 2002

122 **D'Amico G**, Garcia-Tsao G, Pagliaro L. Natural history and prognostic indicators of survival in cirrhosis: a systematic review of 118 studies. *J Hepatol* 2006; **44**: 217-231 [PMID: 16298014 DOI: 10.1016/j.jhep.2005.10.013]

123 **Cillo U**, Amodio P, Ronco C, Soni SS, Zanus G, Minazzato L, Salari A, Neri D, Bombonato G, Schiff S, Bianco T. Hepatitis C virus adversely affects quality of life. *Blood Purif* 2011; **32**: 144-149 [PMID: 21659741 DOI: 10.1159/000325222]

124 **Dharancy S**, Lemyze M, Boleslawski E, Neviere R, Declerck N, Canva V, Wallaert B, Mathurin P, Pruvot FR. Impact of impaired aerobic capacity on liver transplant candidates. *Transplantation* 2008; **86**: 1077-1083 [PMID: 18946345 DOI: 10.1097/TP.0b013e318187758b]

125 **Foroncewicz B**, Mucha K, Szparaga B, Raczyńska J, Ciszek M, Pilecki T, Krawczyk M, Pączek L. Rehabilitation and 6-minute walk test after liver transplantation. *Transplant Proc* 2011; **43**: 3021-3024 [PMID: 21996215 DOI: 10.1016/j.transproceed.2011.08.007]

126 **Faustini-Pereira JL**, Homercher-Galant L, Garcia E, de Mello Brandão AB, Marroni CA. Exercise capacity of cirrhotic patients with hepatopulmonary syndrome. *Ann Hepatol* 2015; **14**: 361-368 [PMID: 25864217]

127 **Jacobsen EB**, Hamberg O, Quistorff B, Ott P. Reduced mitochondrial adenosine triphosphate synthesis in skeletal muscle in patients with Child-Pugh class B and C cirrhosis. *Hepatology* 2001; **34**: 7-12 [PMID: 11431727 DOI: 10.1053/jhep.2001.25451]

128 **Montano-Loza AJ**, Meza-Junco J, Prado CM, Lieffers JR, Baracos VE, Bain VG, Sawyer MB. Muscle wasting is associated with mortality in patients with cirrhosis. *Clin Gastroenterol Hepatol* 2012; **10**: 166-173, 173.e1 [PMID: 21893129 DOI: 10.1016/

j.cgh.2011.08.028]

129 **Montano-Loza AJ**. New concepts in liver cirrhosis: clinical significance of sarcopenia in cirrhotic patients. *Minerva Gastroenterol Dietol* 2013; **59**: 173-186 [PMID: 23831908]

130 **Montano-Loza AJ,** Meza-Junco J, Prado CMM, Tandon P, Bain VG, Ma M, Beaumont C, Esfandiari N, Sawyer MB, Baracos VE. New cutoff values for sarcopenia for predicting 6-months mortality in cirrhotic patients. *J Hepatol* 2013; 58: S95 [DOI: 10.1016/S0168-8278(13)60223-8]

131 **Galant LH**, Forgiarini Junior LA, Dias AS, Marroni CA. Functional status, respiratory muscle strength, and quality of life in patients with cirrhosis. *Rev Bras Fisioter* 2012; **16**: 30-34 [PMID: 22441225 DOI: 10.1590/S1413-35552012000100006]

132 **Faustini Pereira JL**, Galant LH, Rossi D, Telles da Rosa LH, Garcia E, de Mello Brandão AB, Marroni CA. Functional Capacity, Respiratory Muscle Strength, and Oxygen Consumption Predict Mortality in Patients with Cirrhosis. *Can J Gastroenterol Hepatol* 2016; **2016**: 6940374 [PMID: 27559536 DOI: 10.1155/2016/6940374]

133 **Galant LH**, Forgiarini Junior LA, Dias AS, Marroni CA. Maximum oxygen consumption predicts mortality in patients with alcoholic cirrhosis. *Hepatogastroenterology* 2013; **60**: 1127-1130 [PMID: 23425809]

134 **Trivedi HD**, Tapper EB. Interventions to improve physical function and prevent adverse events in cirrhosis. *Gastroenterol Rep (Oxf)* 2018; **6**: 13-20 [PMID: 29479438 DOI: 10.1093/gastro/gox042]

135 **Martin P**, DiMartini A, Feng S, Brown R Jr, Fallon M. Evaluation for liver transplantation in adults: 2013 practice guideline by the American Association for the Study of Liver Diseases and the American Society of Transplantation. *Hepatology* 2014; **59**: 1144-1165 [PMID: 24716201 DOI: 10.1002/hep.26972]

136 **Ursic-Bedoya J**, Faure S, Donnadieu-Rigole H, Pageaux GP. Liver transplantation for alcoholic liver disease: Lessons learned and unresolved issues. *World J Gastroenterol* 2015; **21**: 10994-11002 [PMID: 26494956 DOI: 10.3748/wjg.v21.i39.10994]

137 **Ewing JA**. Detecting alcoholism. THE CAGE questionnaire. *JAMA* 1984; **252**: 1905-1907 [PMID: 6471323 DOI: 10.1001/jama.1984.03350140051025]

138 **Saunders JB**, Aasland OG, Babor TF, de la Fuente JR, Grant M. Development of the Alcohol Use Disorders Identification Test (AUDIT): WHO Collaborative Project on Early Detection of Persons with Harmful Alcohol Consumption--II. *Addiction* 1993; **88**: 791-804 [PMID: 8329970 DOI: 10.1111/j.1360-0443.1993.tb02093.x]

139 **Maldonado JR**, Dubois HC, David EE, Sher Y, Lolak S, Dyal J, Witten D. The Stanford Integrated Psychosocial Assessment for Transplantation (SIPAT): a new tool for the psychosocial evaluation of pre-transplant candidates. *Psychosomatics* 2012; **53**: 123-132 [PMID: 22424160 DOI: 10.1016/j.psym.2011.12.012]

140 **Hoofnagle JH**, Kresina T, Fuller RK, Lake JR, Lucey MR, Sorrell MF, Beresford TP. Liver transplantation for alcoholic liver disease: executive statement and recommendations. Summary of a National Institutes of Health workshop held December 6-7, 1996, Bethesda, Maryland. *Liver Transpl Surg* 1997; **3**: 347-350 [PMID: 9346762 DOI: 10.1002/lt.500030324]

141 **Lucey MR**. Issues in selection for and outcome of liver transplantation in patients with alcoholic liver disease. *Liver Transpl Surg* 1997; **3**: 227-230 [PMID: 9346744 DOI: 10.1002/lt.500030306]

142 **DiMartini A**, Day N, Dew MA, Javed L, Fitzgerald MG, Jain A, Fung JJ, Fontes P. Alcohol consumption patterns and predictors of use following liver transplantation for alcoholic liver disease. *Liver Transpl* 2006; **12**: 813-820 [PMID: 16528710 DOI: 10.1002/lt.20688]

143 **Day E**, Best D, Sweeting R, Russell R, Webb K, Georgiou G, Neuberger J. Detecting lifetime alcohol problems in individuals referred for liver transplantation for nonalcoholic liver failure. *Liver Transpl* 2008; **14**: 1609-1613 [PMID: 18975295 DOI: 10.1002/lt.21528]

144 **Singal AK**, Chaha KS, Rasheed K, Anand BS. Liver transplantation in alcoholic liver disease current status and controversies. *World J Gastroenterol* 2013; **19**: 5953-5963 [PMID: 24106395 DOI: 10.3748/wjg.v19.i36.5953]

145 **Boffetta P**, Hashibe M. Alcohol and cancer. *Lancet Oncol* 2006; **7**: 149-156 [PMID: 16455479 DOI: 10.1016/S1470-2045(06)70577-0]

146 **Torok NJ**. Update on Alcoholic Hepatitis. *Biomolecules* 2015; **5**: 2978-2986 [PMID: 26540078 DOI: 10.3390/biom5042978]

147 **Lucey MR**, Mathurin P, Morgan TR. Alcoholic hepatitis. *N Engl J Med* 2009; **360**: 2758-2769 [PMID: 19553649 DOI: 10.1056/NEJMra0805786]

148 **Mendenhall CL**, Moritz TE, Roselle GA, Morgan TR, Nemchausky BA, Tamburro CH, Schiff ER, McClain CJ, Marsano LS, Allen JI. A study of oral nutritional support with oxandrolone in malnourished patients with alcoholic hepatitis: results of a Department of Veterans Affairs cooperative study. *Hepatology* 1993; **17**: 564-576 [PMID: 8477961 DOI: 10.1002/hep.1840170407]

149 **Naveau S**, Giraud V, Borotto E, Aubert A, Capron F, Chaput JC. Excess weight risk factor for alcoholic liver disease. *Hepatology* 1997; **25**: 108-111 [PMID: 8985274 DOI: 10.1002/hep.510250120]

150 **Cohen SM**, Ahn J. Review article: the diagnosis and management of alcoholic hepatitis. *Aliment Pharmacol Ther* 2009; **30**: 3-13 [PMID: 19416132 DOI: 10.1111/j.1365-2036.2009.04002.x]

151 **Mendenhall CL**. Alcoholic hepatitis. *Clin Gastroenterol* 1981; **10**: 417-441 [PMID: 7018751]

152 **Cohen JA**, Kaplan MM. The SGOT/SGPT ratio--an indicator of alcoholic liver disease. *Dig Dis Sci* 1979; **24**: 835-838 [PMID: 520102 DOI: 10.1007/BF01324898]

153 **Sorbi D**, Boynton J, Lindor KD. The ratio of aspartate aminotransferase to alanine aminotransferase: potential value in differentiating nonalcoholic steatohepatitis from alcoholic liver disease. *Am J Gastroenterol* 1999; **94**: 1018-1022 [PMID: 10201476 DOI: 10.1111/j.1572-0241.1999.01006.x]

154 **Williams AL**, Hoofnagle JH. Ratio of serum aspartate to alanine aminotransferase in chronic hepatitis. Relationship to cirrhosis. *Gastroenterology* 1988; **95**: 734-739 [PMID: 3135226 DOI: 10.1016/S0016-5085(88)80022-2]

155 **Altamirano J**, Miquel R, Katoonizadeh A, Abraldes JG, Duarte-Rojo A, Louvet A, Augustin S, Mookerjee RP, Michelena J, Smyrk TC, Buob D, Leteurtre E, Rincón D, Ruiz P, García-Pagán JC, Guerrero-Marquez C, Jones PD, Barritt AS 4th, Arroyo V, Bruguera M, Bañares R, Ginès P, Caballería J, Roskams T, Nevens F, Jalan R, Mathurin P, Shah VH, Bataller R. A histologic scoring system for prognosis of patients with alcoholic hepatitis. *Gastroenterology* 2014; **146**: 1231-9.e1-e6 [PMID: 24440674 DOI: 10.1053/j.gastro.2014.01.018]

156 **Ishak KG**, Zimmerman HJ, Ray MB. Alcoholic liver disease: pathologic, pathogenetic and clinical aspects. *Alcohol Clin Exp Res* 1991; **15**: 45-66 [PMID: 2059245 DOI: 10.1111/j.1530-0277.1991.tb00518.x]

157 **Lefkowitch JH**. Morphology of alcoholic liver disease. *Clin Liver Dis* 2005; **9**: 37-53 [PMID: 15763228 DOI: 10.1016/j.cld.2004.11.001]

158 **Louvet A**, Naveau S, Abdelnour M, Ramond MJ, Diaz E, Fartoux L, Dharancy S, Texier F, Hollebecque A, Serfaty L, Boleslawski E, Deltenre P, Canva V, Pruvot FR, Mathurin P. The Lille model: a new tool for therapeutic strategy in patients with severe alcoholic hepatitis treated with steroids. *Hepatology* 2007; **45**: 1348-1354 [PMID: 17518367 DOI: 10.1002/hep.21607]

159 **Dominguez M**, Rincón D, Abraldes JG, Miquel R, Colmenero J, Bellot P, García-Pagán JC, Fernández R, Moreno M, Bañares R, Arroyo V, Caballería J, Ginès P, Bataller R. A new scoring system for prognostic stratification of patients with alcoholic hepatitis. *Am J Gastroenterol* 2008; **103**: 2747-2756 [PMID: 18721242 DOI: 10.1111/j.1572-0241.2008.02104.x]

160 **Imperiale TF**, McCullough AJ. Do corticosteroids reduce mortality from alcoholic hepatitis? A meta-analysis of the randomized trials. *Ann Intern Med* 1990; **113**: 299-307 [PMID: 2142869 DOI: 10.7326/0003-4819-113-4-299]

161 **Maddrey WC**, Boitnott JK, Bedine MS, Weber FL Jr, Mezey E, White RI Jr. Corticosteroid therapy of alcoholic hepatitis. *Gastroenterology* 1978; **75**: 193-199 [PMID: 352788]

162 **Carithers RL Jr**, Herlong HF, Diehl AM, Shaw EW, Combes B, Fallon HJ, Maddrey WC. Methylprednisolone therapy in patients

with severe alcoholic hepatitis. A randomized multicenter trial. *Ann Intern Med* 1989; **110**: 685-690 [PMID: 2648927 DOI: 10.7326/0003-4819-110-9-685]

163   **Phillips M**, Curtis H, Portmann B, Donaldson N, Bomford A, O'Grady J. Antioxidants versus corticosteroids in the treatment of severe alcoholic hepatitis--a randomised clinical trial. *J Hepatol* 2006; **44**: 784-790 [PMID: 16469404 DOI: 10.1016/j.jhep.2005.11.039]

164   **Friedman SL**. Management and prognosis of alcoholic hepatitis. In: Runyon BA, Robson KM, eds. UpToDate. Available from: URL: https://www.uptodate.com/contents/management-and-prognosis-of-alcoholic-hepatitis

165   **Sheth M**, Riggs M, Patel T. Utility of the Mayo End-Stage Liver Disease (MELD) score in assessing prognosis of patients with alcoholic hepatitis. *BMC Gastroenterol* 2002; **2**: 2 [PMID: 11835693 DOI: 10.1186/1471-230X-2-2]

166   **Srikureja W**, Kyulo NL, Runyon BA, Hu KQ. MELD score is a better prognostic model than Child-Turcotte-Pugh score or Discriminant Function score in patients with alcoholic hepatitis. *J Hepatol* 2005; **42**: 700-706 [PMID: 15826720 DOI: 10.1016/j.jhep.2004.12.022]

167   **Dunn W**, Jamil LH, Brown LS, Wiesner RH, Kim WR, Menon KV, Malinchoc M, Kamath PS, Shah V. MELD accurately predicts mortality in patients with alcoholic hepatitis. *Hepatology* 2005; **41**: 353-358 [PMID: 15660383 DOI: 10.1002/hep.20503]

168   **Mathurin P**, Lucey MR. Management of alcoholic hepatitis. *J Hepatol* 2012; **56 Suppl 1**: S39-S45 [PMID: 22300464 DOI: 10.1016/S0168-8278(12)60005-1]

169   **O'Shea RS**, Dasarathy S, McCullough AJ. Alcoholic liver disease. *Am J Gastroenterol* 2010; **105**: 14-32; quiz 33 [PMID: 19904248 DOI: 10.1038/ajg.2009.593]

170   **Louvet A**, Wartel F, Castel H, Dharancy S, Hollebecque A, Canva-Delcambre V, Deltenre P, Mathurin P. Infection in patients with severe alcoholic hepatitis treated with steroids: early response to therapy is the key factor. *Gastroenterology* 2009; **137**: 541-548 [PMID: 19445945 DOI: 10.1053/j.gastro.2009.04.062]

171   **Mathurin P**, Mendenhall CL, Carithers RL Jr, Ramond MJ, Maddrey WC, Garstide P, Rueff B, Naveau S, Chaput JC, Poynard T. Corticosteroids improve short-term survival in patients with severe alcoholic hepatitis (AH): individual data analysis of the last three randomized placebo controlled double blind trials of corticosteroids in severe AH. *J Hepatol* 2002; **36**: 480-487 [PMID: 11943418 DOI: 10.1016/S0168-8278(01)00289-6]

172   **O'Shea RS**, Dasarathy S, McCullough AJ; Practice Guideline Committee of the American Association for the Study of Liver Diseases; Practice Parameters Committee of the American College of Gastroenterology. Alcoholic liver disease. *Hepatology* 2010; **51**: 307-328 [PMID: 20034030 DOI: 10.1002/hep.23258]

173   **Thursz MR**, Richardson P, Allison M, Austin A, Bowers M, Day CP, Downs N, Gleeson D, MacGilchrist A, Grant A, Hood S, Masson S, McCune A, Mellor J, O'Grady J, Patch D, Ratcliffe I, Roderick P, Stanton L, Vergis N, Wright M, Ryder S, Forrest EH; STOPAH Trial. Prednisolone or pentoxifylline for alcoholic hepatitis. *N Engl J Med* 2015; **372**: 1619-1628 [PMID: 25901427 DOI: 10.1056/NEJMoa1412278]

174   **Singh S**, Murad MH, Chandar AK, Bongiorno CM, Singal AK, Atkinson SR, Thursz MR, Loomba R, Shah VH. Comparative Effectiveness of Pharmacological Interventions for Severe Alcoholic Hepatitis: A Systematic Review and Network Meta-analysis. *Gastroenterology* 2015; **149**: 958-70.e12 [PMID: 26091937 DOI: 10.1053/j.gastro.2015.06.006]

175   **Park SH**, Kim DJ, Kim YS, Yim HJ, Tak WY, Lee HJ, Sohn JH, Yoon KT, Kim IH, Kim HS, Um SH, Baik SK, Lee JS, Suk KT, Kim SG, Suh SJ, Park SY, Kim TY, Jang JY; Korean Association for the Study of the Liver (KASL)-Alcohol Related Problems Study Group. Pentoxifylline vs. corticosteroid to treat severe alcoholic hepatitis: a randomised, non-inferiority, open trial. *J Hepatol* 2014; **61**: 792-798 [PMID: 24845609 DOI: 10.1016/j.jhep.2014.05.014]

176   **Mathurin P**, Louvet A, Duhamel A, Nahon P, Carbonell N, Boursier J, Anty R, Diaz E, Thabut D, Moirand R, Lebrec D, Moreno C,

Talbodec N, Paupard T, Naveau S, Silvain C, Pageaux GP, Sobesky R, Canva-Delcambre V, Dharancy S, Salleron J, Dao T. Prednisolone with vs without pentoxifylline and survival of patients with severe alcoholic hepatitis: a randomized clinical trial. *JAMA* 2013; **310**: 1033-1041 [PMID: 24026598 DOI: 10.1001/jama.2013.276300]

177   **Yu CH**, Xu CF, Ye H, Li L, Li YM. Early mortality of alcoholic hepatitis: a review of data from placebo-controlled clinical trials. *World J Gastroenterol* 2010; **16**: 2435-2439 [PMID: 20480532 DOI: 10.3748/wjg.v16.i19.2435]

178   **Mathurin P**, O'Grady J, Carithers RL, Phillips M, Louvet A, Mendenhall CL, Ramond MJ, Naveau S, Maddrey WC, Morgan TR. Corticosteroids improve short-term survival in patients with severe alcoholic hepatitis: meta-analysis of individual patient data. *Gut* 2011; **60**: 255-260 [PMID: 20940288 DOI: 10.1136/gut.2010.224097]

179   **Akriviadis E**, Botla R, Briggs W, Han S, Reynolds T, Shakil O. Pentoxifylline improves short-term survival in severe acute alcoholic hepatitis: a double-blind, placebo-controlled trial. *Gastroenterology* 2000; **119**: 1637-1648 [PMID: 11113085 DOI: 10.1053/gast.2000.20189]

180   **Simon D**, Galambos JT. A randomized controlled study of peripheral parenteral nutrition in moderate and severe alcoholic hepatitis. *J Hepatol* 1988; **7**: 200-207 [PMID: 3142949 DOI: 10.1016/S0168-8278(88)80483-5]

181   **Mezey E**, Potter JJ, Rennie-Tankersley L, Caballeria J, Pares A. A randomized placebo controlled trial of vitamin E for alcoholic hepatitis. *J Hepatol* 2004; **40**: 40-46 [PMID: 14672612 DOI: 10.1016/S0168-8278(03)00476-8]

182   **Potts JR**, Goubet S, Heneghan MA, Verma S. Determinants of long-term outcome in severe alcoholic hepatitis. *Aliment Pharmacol Ther* 2013; **38**: 584-595 [PMID: 23879720 DOI: 10.1111/apt.12427]

183   **Singal AK**, Kamath PS, Gores GJ, Shah VH. Alcoholic hepatitis: current challenges and future directions. *Clin Gastroenterol Hepatol* 2014; **12**: 555-564; quiz e31-e32 [PMID: 23811249 DOI: 10.1016/j.cgh.2013.06.013]

184   **Mathurin P**, Moreno C, Samuel D, Dumortier J, Salleron J, Durand F, Castel H, Duhamel A, Pageaux GP, Leroy V, Dharancy S, Louvet A, Boleslawski E, Lucidi V, Gustot T, Francoz C, Letoublon C, Castaing D, Belghiti J, Donckier V, Pruvot FR, Duclos-Vallée JC. Early liver transplantation for severe alcoholic hepatitis. *N Engl J Med* 2011; **365**: 1790-1800 [PMID: 22070476 DOI: 10.1056/NEJMoa1105703]

185   **Singal AK**, Bashar H, Anand BS, Jampana SC, Singal V, Kuo YF. Outcomes after liver transplantation for alcoholic hepatitis are similar to alcoholic cirrhosis: exploratory analysis from the UNOS database. *Hepatology* 2012; **55**: 1398-1405 [PMID: 22213344 DOI: 10.1002/hep.25544]

186   **Im GY**, Kim-Schluger L, Shenoy A, Schubert E, Goel A, Friedman SL, Florman S, Schiano TD. Early Liver Transplantation for Severe Alcoholic Hepatitis in the United States--A Single-Center Experience. *Am J Transplant* 2016; **16**: 841-849 [PMID: 26710309 DOI: 10.1111/ajt.13586]

187   **Lee BP**, Chen PH, Haugen C, Hernaez R, Gurakar A, Philosophe B, Dagher N, Moore SA, Li Z, Cameron AM. Three-year Results of a Pilot Program in Early Liver Transplantation for Severe Alcoholic Hepatitis. *Ann Surg* 2017; **265**: 20-29 [PMID: 27280501 DOI: 10.1097/SLA.0000000000001831]

188   **Marot A**, Dubois M, Trépo E, Moreno C, Deltenre P. Liver transplantation for alcoholic hepatitis: A systematic review with meta-analysis. *PLoS One* 2018; **13**: e0190823 [PMID: 29324766 DOI: 10.1371/journal.pone.0190823]

189   **Kubiliun M**, Patel SJ, Hur C, Dienstag JL, Luther J. Early liver transplantation for alcoholic hepatitis: Ready for primetime? *J Hepatol* 2018; **68**: 380-382 [PMID: 29175244 DOI: 10.1016/j.jhep.2017.11.027]

190   **Singal AK**, Bataller R, Ahn J, Kamath PS, Shah VH. ACG Clinical Guideline: Alcoholic Liver Disease. *Am J Gastroenterol* 2018; **113**: 175-194 [PMID: 29336434 DOI: 10.1038/ajg.2017.469]

191   **Litten RZ**, Bradley AM, Moss HB. Alcohol biomarkers in applied settings: recent advances and future research opportunities. *Alcohol Clin Exp Res* 2010; **34**: 955-967 [PMID: 20374219 DOI: 10.1111/j.1530-0277.2010.01170.x]

192 **Staufer K**, Andresen H, Vettorazzi E, Tobias N, Nashan B, Sterneck M. Urinary ethyl glucuronide as a novel screening tool in patients pre- and post-liver transplantation improves detection of alcohol consumption. *Hepatology* 2011; **54**: 1640-1649 [PMID: 21809364 DOI: 10.1002/hep.24596]

193 **Sterneck M**, Yegles M, Rothkirch von G, Staufer K, Vettorazzi E, Schulz KH, Tobias N, Graeser C, Fischer L, Nashan B, Andresen-Streichert H. Determination of ethyl glucuronide in hair improves evaluation of long-term alcohol abstention in liver transplant candidates. *Liver Int* 2014; **34**: 469-476 [PMID: 23829409 DOI: 10.1111/liv.12243]

194 **DiMartini A**, Dew MA, Day N, Fitzgerald MG, Jones BL, deVera ME, Fontes P. Trajectories of alcohol consumption following liver transplantation. *Am J Transplant* 2010; **10**: 2305-2312 [PMID: 20726963 DOI: 10.1111/j.1600-6143.2010.03232.x]

195 **Dumortier J**, Dharancy S, Cannesson A, Lassailly G, Rolland B, Pruvot FR, Boillot O, Faure S, Guillaud O, Rigole-Donnadieu H, Herrero A, Scoazec JY, Mathurin P, Pageaux GP. Recurrent alcoholic cirrhosis in severe alcoholic relapse after liver transplantation:

a frequent and serious complication. *Am J Gastroenterol* 2015; **110**: 1160-1166; quiz 1167 [PMID: 26169514 DOI: 10.1038/ajg.2015.204]

196 **Wigg AJ**, Mangira D, Chen JW, Woodman RW. Outcomes and predictors of harmful relapse following liver transplantation for alcoholic liver disease in an Australian population. *Intern Med J* 2017; **47**: 656-663 [PMID: 28321963 DOI: 10.1111/imj.13431]

197 **Cheung A**, Levitsky J. Follow-up of the Post-Liver Transplantation Patient: A Primer for the Practicing Gastroenterologist. *Clin Liver Dis* 2017; **21**: 793-813 [PMID: 28987263 DOI: 10.1016/j.cld.2017.06.006]

198 **Pischke S**, Lege MC, von Wulffen M, Galante A, Otto B, Wehmeyer MH, Herden U, Fischer L, Nashan B, Lohse AW, Sterneck M. Factors associated with long-term survival after liver transplantation: A retrospective cohort study. *World J Hepatol* 2017; **9**: 427-435 [PMID: 28357030 DOI: 10.4254/wjh.v9.i8.427]

199 **Zeair S**, Cyprys S, Wiśniewska H, Bugajska K, Parczewski M, Wawrzynowicz-Syczewska M. Alcohol Relapse After Liver Transplantation: Younger Women Are at Greatest Risk. *Ann Transplant* 2017; **22**: 725-729 [PMID: 29208851 DOI: 10.12659/AOT.905335]

REVIEW

Current clinical management of gastrointestinal stromal tumor

Kazuya Akahoshi, Masafumi Oya, Tadashi Koga, Yuki Shiratsuchi

Kazuya Akahoshi, Yuki Shiratsuchi, Department of Gastroenterology, Aso Iizuka Hospital, Iizuka 820-8505, Japan

Masafumi Oya, Department of Pathology, Aso Iizuka Hospital, Iizuka 820-8505, Japan

Tadashi Koga, Department of Surgery, Aso Iizuka Hospital, Iizuka 820-8505, Japan

ORCID number: Kazuya Akahoshi (0000-0002-1095-6546); Masafumi Oya (0000-0003-0421-5044); Tadashi Koga (0000-0002-6420-6214); Yuki Shiratsuchi (0000-0003-0085-8683).

Abstract

Gastrointestinal stromal tumors (GISTs) are the most common malignant subepithelial lesions (SELs) of the gastrointestinal tract. They originate from the interstitial cells of Cajal located within the muscle layer and are characterized by over-expression of the tyrosine kinase receptor KIT. Pathologically, diagnosis of a GIST relies on morphology and immunohistochemistry [KIT and/or discovered on gastrointestinal stromal tumor 1 (DOG1) is generally positive]. The prognosis of this disease is associated with the tumor size and mitotic index. The standard treatment of a GIST without metastasis is surgical resection. A GIST with metastasis is usually only treated by tyrosine kinase inhibitors without radical cure; thus, early diagnosis is the only way to improve its prognosis. However, a GIST is usually detected as a SEL during endoscopy, and many benign and malignant conditions may manifest as SELs. Conventional endoscopic biopsy is difficult for tumors without ulceration. Most SELs have therefore been managed without a histological diagnosis. However, a favorable prognosis of a GIST is associated with early histological diagnosis and R0 resection. Endoscopic ultrasonography (EUS) and EUS-guided fine needle aspiration (EUS-FNA) are critical for an accurate diagnosis of SELs. EUS-FNA is safe and effective in enabling an early histological diagnosis and adequate treatment. This review outlines the current evidence for the diagnosis and management of GISTs, with an emphasis on early management of small SELs.

Key words: Gastrointestinal stromal tumor; Endoscopic ultrasonography-guided fine needle aspiration; Endoscopic ultrasonography; Diagnosis; Therapy

Core tip: Potentially malignant gastrointestinal stromal tumors are the most common subepithelial lesions (SELs) of the gastrointestinal tract. SELs include a broader range of differential diagnoses from benign to malignant lesions.

The possibility of having a malignant lesion may cause anxiety and discomfort in patients and gastroenterologists. Early and accurate diagnosis of SELs using endoscopic ultrasonography (EUS) and/or EUS-guided fine needle aspiration is vital to guide selection of early appropriate management.

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common malignant subepithelial lesions (SELs) of the gastrointestinal tract in the daily clinical setting[1,2]. GISTs are thought to originate from the interstitial cells of Cajal, which are the pacemaker cells of gastrointestinal movement[3]. GISTs are largely caused by oncogenic mutations in the tyrosine kinase receptor KIT[4] and/or platelet-derived growth factor receptor-α (PDGFR-α)[5]. Approximately 10% to 30% of GISTs have a malignant clinical course[1,6,7]. Additionally, it has been reported that not only large GISTs with a high mitotic index frequently exhibit a malignant clinical course, but also small GISTs with a low mitotic index rarely show a malignant course with metastasis. Thus, a GIST is considered to be a potentially malignant tumor. GISTs are not classified as either benign or malignant but are rather stratified by their clinical risk of malignancy: Very low, low, intermediate, or high[7]. Mietinenn reported that the metastatic risk of GISTs increases according to the tumor size irrespective of the mitotic count[6] (Figure 1). Surgical resection is the primary approach to management of localized GISTs[8]. Despite complete resection, postoperative recurrence occurs in at least half of all patients with GISTs[2,9]. Although tyrosine kinase inhibitors have been shown to provide sustained disease management in patients with metastasis[10-16], surgical R0 resection of small GISTs without metastasis is the only promising treatment for a permanent cure[8,17]. The best treatment strategy for GISTs is early diagnosis and early resection. However, GISTs are frequently detected as SELs during endoscopy[8,18-20]. The differential diagnoses of SELs are quite broad and can include extra-gastrointestinal tract compression, varices, an ectopic pancreas, and various tumors including GIST, SEL-like cancer, leiomyoma, schwannoma, and lipoma[8,20,21]. GISTs should be diagnosed by immunohistochemical analysis including assessment of KIT, CD34, and/or discovered on gastrointestinal stromal tumor 1 (DOG1)[8,22,23]. However, it is more difficult to obtain a conclusive histologic diagnosis of a GIST than gastrointestinal cancer by standard endoscopic forceps biopsy because a GIST is covered by normal mucosa. Although imaging tests including endoscopic ultrasonography (EUS) and computed tomography (CT) are useful for narrowing down the differential diagnoses of SELs, these techniques are unable to provide a conclusive diagnosis. At present, EUS-guided fine needle aspiration (EUS-FNA) is the most accurate, safe, and reliable preoperative immunohistological test to secure a definitive diagnosis of SELs[8,18,19,23]. Aggressive use of EUS and EUS-FNA for SELs is the key to facilitating early intervention of GISTs[21,23].

This paper provides an overview of the diagnosis and treatment of GISTs, with an emphasis on early diagnosis and management of GISTs using EUS-FNA.

EPIDEMIOLOGY

In epidemiological surveys of GISTs, the estimated mean age at diagnosis is in the sixth decade of life, and the frequency of occurrence is 6.8 to 14.5 cases per million individuals per year[24-26]. GISTs most commonly occur in the stomach (51%), followed by the small intestine (36%), colon (7%), rectum (5%), and esophagus (1%)[24].

HISTOLOGICAL FINDINGS

The main morphologic types of GISTs are the spindle-shaped cell type (70%), epithelial cell type (20%), and mixed type (10%)[27]. It is difficult to differentiate between leiomyomas and neurinomas, two other mesenchymal tumors, using only hematoxylin and eosin staining; differentiation using immunostaining is indispensable[8,22,23]. A GIST is diagnosed in the presence of KIT or CD34 positivity. If the tumor is negative for KIT, CD34, desmin, and S-100, additional tests including DOG1 staining or a mutation search of the KIT or PDGFRA gene are useful for diagnosis of GISTs[28] (Figure 2).

CLINICAL PRESENTATION AND INCIDENTAL GIST

The most common symptoms of GISTs are gastrointestinal bleeding, including acute melena and hematemesis with subsequent anemia; weakness; and abdominal pain, distension, and discomfort due to a tumor-induced mass effect[29]. Previous studies have shown that 15% to 30% of patients with GISTs are asymptomatic, and their GISTs are found incidentally during postmortem autopsy or surgery for treatment of other diseases[6,25,30]. Many pathological studies have highlighted the existence of subclinical microscopic or so-called mini (< 1 cm) GISTs[31-36]. Kawanowa *et al*[31] reported that microscopic GISTs were present in 35% of patients who underwent gastrectomy for treatment of gastric adenocarcinoma. Agaimy *et al*[32] reported that microscopic gastric GISTs were found in 22.5% of consecutive autopsies of patients aged $\geqslant$ 50 years. The reported incidence of mini-GISTs according to the affected organ is 3% to 10% in the stomach, 0.2% in the colon, and 0.01% in the

Figure 1 Rate of metastasis or tumor-related death according to tumor diameter and mitotic index. Created using reference[6].

Figure 2 Flow chart of diagnosis of gastrointestinal mesenchymal tumors using immunohistochemical or genetic analysis. [1]Solitary fibrous tumors should be ruled out. Quoted and modified from reference[8]. GIST: Gastrointestinal stromal tumor.

rectum[31-36]. The detection of incidental SELs during gastrointestinal endoscopy has recently increased with the more widespread performance of endoscopic examinations. Gastric SELs are found in 0.36% of middle-aged adults during health examinations, and half of these tumors are considered to be neoplastic[37]. Most gastric SELs found during physical check-ups are small and asymptomatic. GISTs are considered to account for half of these incidentally found SELs in the stomach[31,34]. Based on these studies, GISTs are presumed to be much more common than previously recognized[18].

ENDOSCOPY

SELs are frequently found during ordinary optical endoscopy. The main endoscopic finding of GISTs is common to all SELs: A nonspecific smooth bulge covered with normal mucosa[11,21,38] (Figure 3). Therefore, endoscopic examination provides insufficient information for differential diagnosis of SELs. Irregular borders, ulceration, and/or growth during endoscopic follow-up are considered clinically malignant features on endoscopy[36]. GISTs are usually hard and the cushion sign is negative.

When GISTs increase in size, ulceration may be seen on the top of the tumor[19].

EUS

EUS is a key test for differential diagnosis of SELs because it provides high-resolution tomographic imaging using high-frequency ultrasound. EUS provides the following information regarding SELs[39] (Figure 4): The gastrointestinal wall layer from which it originates (within the submucosal layer, in continuity with the muscularis propria, or outside the wall), the nature of the lesion (liquid, fat, solid tumor, or blood vessel), and the true size of the SEL from a cross-sectional image[39]. Thus, EUS is the safest and most useful modality for differential diagnosis and follow-up of SELs[21,40,41]. EUS allows for the conclusive diagnosis of many lesions using echo findings only, such as lipomas (highly echoic masses) (Figure 3A and B), cysts (anechoic masses) (Figure 3C and D), extraluminal compression by surrounding normal organs or lesions[42] (Figure 3E and F), and varices (Figure 3G and H)[21,38,43]. The typical EUS imaging feature of a GIST is a hypoechoic solid mass. EUS can accurately discriminate a SEL suspected to be a GIST (hypoechoic solid mass) from other SELs, including lipomas, cysts, varices, and extra-gastrointestinal compression. According to previous reports, possible high-risk EUS features for GISTs are a size of > 2 cm, irregular borders, heterogeneous echo patterns, anechoic spaces, echogenic foci, and growth during follow-up[44,45]. However, Kim *et al*[46] reported that tumor size and EUS features cannot be used to preoperatively predict the risk of malignancy of medium-sized (2-5 cm) gastric GISTs. At present, estimation of the risk of malignancy of GISTs of < 5 cm by EUS imaging alone seems to be difficult. The finding of a hypoechoic solid mass by EUS is also seen in malignant tumors such as malignant lymphoma, metastatic cancer, neuroendocrine tumor, and SEL-like cancer and in benign conditions such as leiomyoma, neurinoma, and an aberrant pancreas[21]. It is difficult to distinguish among these lesions using EUS findings only. The accuracy of differential diagnosis of SELs by EUS is extremely poor and ranges from 45.5% to 48.0%[47,48]. Because current EUS imaging characteristics alone provide insufficient accuracy in the diagnosis of GISTs, tissue sampling for immunohistochemical analysis using EUS-FNA or biopsy is required for a definite diagnosis before surgery or chemotherapy[18-21].

TUMOR TISSUE SAMPLING METHODS

Endoscopic forceps biopsy
Conventional endoscopic forceps biopsy is limited because these forceps usually cannot reach the tumor beyond the overlying normal mucosa and submucosa[49]. When ulceration is present, a biopsy within the ulcer is effective for a conclusive diagnosis[18,49,50]. Although special methods such as "jumbo" or "bite-on-bite" biopsy

Figure 3 Endoscopic images of subepithelial lesions that can be diagnosed only with endoscopic ultrasound findings and their specific endoscopic ultrasonography images. A: Endoscopic image of a gastric lipoma (arrow); B: Endoscopic ultrasound (EUS) image of A (high-echo mass); C: Endoscopic image of a gastric cyst; D: EUS image of C (anechoic mass); E: Endoscopic image of extra-gastric compression due to splenic artery aneurysm; F: EUS image of E [normal gastric wall is compressed by a splenic artery aneurysm(SAA) (arrow). SA: splenic artery]; G: Endoscopic image of gastric varices (arrow); H: EUS image of G [varices are present in the submucosa from the outside of the wall (V) (arrow)]. Quoted and modified from reference[38] with permission.

Figure 4 Differential diagnosis of subepithelial lesions by endoscopic ultrasound. Quoted and modified from reference[39] with permission. GIST: Gastrointestinal stromal tumor.

are available, the diagnostic yield of these approaches is poor, ranging from 17% to 59%[51-53]. Additionally, one study showed that significant bleeding occurred in 35.7% of patients after jumbo biopsy, and 34.9% of patients needed subsequent endoscopic hemostasis[53].

EUS-FNA

EUS-FNA is the most established tissue sampling method for SELs and can provide a conclusive immunohistochemical diagnosis safely and accurately (Figure 5) (Video 1). Typical EUS-FNA findings of GISTs are KIT- or CD34-positive spindle-shaped cells or epithelial cells. The diagnostic rate of SELs using EUS-FNA ranges from 62.0% to 93.4%[23,54-56]. The diagnostic rate according to tumor diameter is 71% for 1-cm to 2-cm tumors,

86% for 2-cm to 4-cm tumors, and 100% for > 4-cm tumors[23]. The diagnostic rate tends to be higher as the tumor diameter increases. Unfortunately, EUS-FNA for a subepithelial hypoechoic solid mass of < 1 cm is technically difficult using a standard EUS-FNA scope; thus, EUS-FNA is recommended for masses of > 1 cm[56,57]. However, forward-viewing and curved linear-array echoendoscopes [58] and drill needles[59] have recently been developed and are expected to improve the diagnostic rate of small SELs. The rate of adverse events associated with EUS-FNA using a 22-gauge needle is reportedly close to 0[54-56].

Evaluation of mitosis is important to determine the metastatic risk of GISTs. Unfortunately, the tissue sample volume obtained by EUS-FNA is usually small. Therefore,

Figure 5 Endoscopic ultrasound images and corresponding endoscopic ultrasonography-guided fine needle aspiration specimens of hypoechoic solid tumors. A: Endoscopic ultrasound (EUS) image of a gastric gastrointestinal stromal tumor; B: EUS-guided fine needle aspiration (EUS-FNA) specimen tissue image of A (KIT-positive spindle-shaped tumor cells are observed); C: EUS image of gastric leiomyoma; D: EUS-FNA specimen tissue image of C [α-SMA-positive spindle-shaped tumor cells are observed; diagnosis of leiomyoma was made by immunohistochemical analysis, which revealed α-SMA (+), KIT (-), CD34 (-), and S-100 (-)]; E: EUS image of gastric malignant lymphoma; F: EUS-FNA specimen image of E (diagnosis of diffuse large B-cell lymphoma was made by CD20-positive lymphoid tumor cells); G: EUS image of rectal neuroendocrine tumor (NET); H: EUS-FNA specimen image of G (diagnosis of NET was made by typical findings of irregular nest of synaptophysin-positive epithelial-like cells). Quoted and modified from reference[38] with permission.

assessment of mitosis by EUS-FNA is difficult. Ando *et al*[60] reported that the MIB-1 labeling index is accurate (100%) for diagnosis of malignant GISTs because Ki-67-positive cells can be easily recognized in the small specimens obtained by EUS-FNA.

Endoscopic biopsy using endoscopic submucosal dissection or endoscopic snare resection techniques
Invasive endoscopic tissue acquisition to obtain a higher tissue volume was recently developed and clinically applied[56-59]. Various endoscopic tissue-obtaining me-

Figure 6 Proposed algorithm for management of subepithelial lesions. Quoted and modified from reference[21]. GIST: Gastrointestinal stromal tumor; GI: Gastrointestinal; SEL: Subepithelial lesion; EUS: Endoscopic ultrasound; EUS-FNA: Endoscopic ultrasound-guided fine needle aspiration.

thods using endoscopic submucosal dissection (ESD) techniques or endoscopic snare resection techniques significantly increase the diagnostic yield when compared with standard forceps biopsy; the reported diagnostic rates range from 85% to 94%[61-64]. An additional advantage of these methods is the ability to evaluate the risk classification of GISTs using the mitotic count per 50 high-power fields[65,66]. However, ESD and endoscopic snare resection are invasive procedures; therefore, endoscopists should pay special attention to intraoperative bleeding and perforation while performing these techniques because such complications may cause severe hypotension or tumor cell seeding. Lee *et al*[63] reported that minor hemorrhage occurred in 56% of patients who underwent endoscopic partial removal with the unroofing technique, but hemostasis was successfully achieved in all patients with argon plasma coagulation, and no perforations occurred. Furthermore, tissue sampling of SELs with an extraluminal growth pattern is difficult[64]. A potential disadvantage of these aggressive endoscopic tissue acquisition techniques using ESD or endoscopic snare resection is the development of perilesional fibrosis, which may render subsequent attempts at submucosal tunneling endoscopic resection[67,68] difficult or even impossible[69].

DIAGNOSTIC PROCESS

GISTs have no specific endoscopic or EUS findings, and diagnosis is difficult to achieve by histopathological examination using hematoxylin and eosin staining alone. Immunohistochemical analysis such as that involving KIT, CD34, or DOG1 measurement is essential for a definitive diagnosis[8,21]. However, because not all SELs are GISTs, it is necessary to identify those SELs that are suspicious for GISTs and perform immunohistochemical analysis of these SELs in clinical practice. Figure 6 shows our institutional algorithm for the detection and management of SELs as discussed

herein[21]. First, all SELs are examined by EUS, and the SELs mentioned in the EUS section (Figure 3) that are conclusively diagnosed by EUS findings only are excluded. Second, EUS-FNA using immunohistochemical analysis is performed for the remaining hypoechoic solid masses to differentiate GISTs from other tumors. Narrowing down of SELs by EUS is important for efficient performance of EUS-FNA in the diagnosis of GISTs. In the Japanese clinical practice guidelines for GISTs, biopsy is recommended for exclusion of SEL-like cancer when an SEL is endoscopically diagnosed[70]. However, because SELs also include vascular diseases for which biopsy is contraindicated, such as varices (Figure 3G and H), it is desirable to perform EUS before biopsy.

TREATMENT

The principle treatment strategy for immunohistologically confirmed GISTs is as follows: (1) Surgical resection is the first choice for resectable GISTs without metastasis; and (2) Administration of tyrosine kinase inhibitors such as imatinib is the primary approach for unresectable, metastatic, or recurrent GISTs[70-73]. The objective of surgery is to achieve R0 resection to the greatest extent possible. Lymph node dissection is not recommended except when lymph node metastasis is clinically suspected; most metastasis of GIST is liver metastasis or peritoneal seeding, and lymph node metastasis is extremely rare[74,75]. Therefore, wedge or segmental resection with preservation of organs and organ functions and maintenance of a good quality of life after surgery is recommended[76]. Previous studies have shown that laparoscopic resection is feasible and safe for gastric GISTs and is less invasive than traditional open surgery, with similar oncological outcomes (Figure 7)[77-79]. Other minimally invasive techniques such as submucosal tunneling endoscopic resection[67,68], endoscopic fullthickness resection[80], and laparoscopic endoscopic cooperative surgery[81] have recently shown good clinical outcomes;

Figure 7 Laparoscopic resection of a small gastric gastrointestinal stromal tumor. A: Laparoscopic view of a small gastric gastrointestinal stromal tumor (arrow) during resection; B: Postoperative endoscopy shows mild postoperative deformity (arrow). Quoted and modified from reference[77] with permission.

Table 1 Modified Fletcher's risk classification

Risk category	Tumor size (cm)	Mitotic index (per 50 HPFs)	Primary tumor site
Very low risk	< 2.0	≤ 5	Any
Low risk	2.1-5.0	≤ 5	Any
Intermediate risk	2.1-5.0	> 5	Gastric
	< 5	6-10	Any
	5.1-10.0	≤ 5	Gastric
High risk	Any	Any	Tumor rupture
	> 10 cm	Any	Any
	Any	> 10	Any
	> 5.0	> 5	Any
	2.1-5.0	> 5	Non-gastric
	5.1-10	≤ 5	Non-gastric

Quoted and modified from reference[7] with permission. HPF: High-power fields.

however, there are still insufficient studies concerning their long-term safety, and they are still at clinical research levels.

In contrast, the introduction of imatinib (first-line tyrosine kinase inhibitor) has dramatically improved the management of GISTs, prolonging recurrence-free survival after surgery[82] and extending overall survival in metastatic or unresectable cases[14]. Three years of adjuvant therapy with imatinib for patients with high-risk GISTs who have undergone macroscopic complete tumor resection (R0 and R1) is recommended because it improves overall survival and recurrence-free survival[82]. Sunitinib (second-line tyrosine kinase inhibitor)[83] and regorafenib (third-line multikinase inhibitor)[84] can be used in advanced GISTs after treatment failure with imatinib. However, it is difficult to obtain a permanent cure by tyrosine kinase inhibitors. Therefore, early diagnosis (early GISTs without metastasis) with early surgical resection is the only promising way to obtain complete cure of this disease[20,21,56].

PROGNOSIS AND RISK CLASSIFICATION

Differentiation between a benign and malignant GIST is difficult even using postoperative histopathological findings. Thus, even if the tumor diameter is small and/or the mitotic rate is low, postoperative metastasis is possible. GISTs are currently regarded as potentially malignant tumors. Discrimination of a benign GIST from a malignant GIST by postoperative histological analysis (tumor diameter, mitotic index, and Ki67 expression level) is difficult; therefore, risk classifications to predict postoperative metastasis have been introduced[7,85,86]. Currently, the modified Fletcher classification (Joensuu classification) is widely used (Table 1)[7]. In addition, contour maps (Figure 8) can be created based on investigation of the prognosis of many cases worldwide. In these maps, the risk of recurrence at the 10th year after surgical treatment of a GIST is calculated using the maximum diameter of the tumor, the number of mitoses, the tumor site, and the presence or absence of tumor capsule rupture; continuous risk assessment is also possible[87]. Using such maps, physicians and patients can predict the probability of recurrence in the 10th postoperative year. This is useful for individual decision-making with respect to adjuvant therapy.

POSTOPERATIVE FOLLOW-UP BY CT

The goal of postoperative follow-up is early detection and management of recurrence. Because the targets of postoperative follow-up observation are local recurrence, liver metastasis, and peritoneal dissemination, abdominal contrast CT, which can be sufficiently evaluated from the diaphragm to the inguinal region, is recommended as a follow-up examination method according to the Japanese clinical practice guideline for GISTs[70]. Based on the above-mentioned risk classification, the following observation intervals are recommended[70]: GISTs with very low, low, and moderate risks are followed up by CT every 6 mo to 1 year, and high-risk and clinically malignant GISTs (those with metastasis, injury to the pseudocapsule, peritoneal dissemination, or infiltration of other organs) are followed up by CT every 4 to 6 mo. The natural history of GISTs is unknown. Previous studies have shown that the estimated 5-year recurrence-free survival rate after surgery is 59.9%; few recurrences occurred after the first 10 years of follow-up[7,88]. Follow-up observation after surgery is considered necessary for

Figure 8 Contour maps for estimating the risk of gastrointestinal stromal tumor recurrence after surgery. Areas of colors according to the recurrence rate at the 10th year after surgical treatment of GIST: Blue-black: 0%-10%, Blue: 10%-20%, Light blue: 20%-40%, Gray: 40%-60%, Pink: 60%-80%, Red: 80%-90%, Dark red: 90%-100%. Reprinted from reference[87] with permission. E-GIST: Extra-gastrointestinal stromal tumor (arising outside the gastrointestinal tract).

more than 10 years.

MANAGEMENT OF SMALL SELS SUSPECTED TO BE GISTS

The detection rate of small GISTs has continuously increased with advancements in endoscopy[89,90]. However, the surveillance and management of GISTs smaller than 2 cm is controversial or lacks evidence-based approaches[41,56,89-92]. Most small GISTs are discovered incidentally and usually show a benign or indolent clinical course. Conversely, strict discrimination between benign and malignant GISTs is considered to be very difficult using both clinical and pathological examinations. Thus, the European Society for Medical Oncology[72], Japanese[70], and Chinese Society of Clinical Oncology[73] GIST guidelines recommend surgical resection when an SEL is immunohistologically diagnosed as a GIST, even when smaller than 2 cm. In contrast, the National Comprehensive Cancer Network[71] guide-

lines recommend that small GISTs of < 2 cm may be periodically followed up by EUS when they lack high-risk features including an irregular border, cystic spaces, ulceration, echogenic foci, and heterogeneity. However, in the examination of 378 histologically diagnosed GISTs of < 2 cm registered in the National Cancer Institute's Surveillance Epidemiology and End Results database, which is a cancer database in the United States, 11.4% of the patients had regional/distant metastatic disease and the 5-year GIST-specific mortality rate was 12.9%[93]. In a study of 43 surgically resected small GISTs of < 2 cm with immunohistochemical analysis, 23% of lesions were classified as having intermediate risk according to the Joensuu risk stratification[56]. Although GISTs of ≤ 2 cm are reportedly metastatic at a low frequency (but not 0%)[89,90,93], early tissue diagnosis and early resection with postoperative follow-up are desired. Importantly, therefore, gastroenterologists should consider early interventions such as EUS for incidentally detected small SELs. Active performance of EUS is effective even

for small SELs of ≤ 2 cm to ensure early detection of hypoechoic solid masses suspected to be GISTs[56]. If EUS imaging of a SEL with an endoscopically negative biopsy shows a hypoechoic solid mass of > 1 cm, subsequent EUS-FNA is needed to obtain a conclusive tissue diagnosis of a GIST[21,56]. Small SELs of < 1 cm are currently recommended to undergo periodic EUS follow-up (every 6 mo or 1 year)[56,91] because EUS-FNA for small SELs of < 1 cm is technically difficult. These aggressive approaches for early diagnosis and early treatment of small SELs, similar to the approaches for gastrointestinal tract cancer, seem to be the only promising way to improve patients' quality of life and prognosis.

CONCLUSION

GISTs are the most common malignant SELs of the digestive tract. According to previous studies, early histologic diagnosis and early surgical resection of small localized disease is currently the most reliable and curative treatment technique for GISTs. However, sufficient prospective studies including small GISTs have not been performed to improve the current clinical GIST management. Further studies are needed to focus on early tissue diagnosis and therapeutic approaches, especially for small SELs.

REFERENCES

1   **Miettinen M**, Lasota J. Gastrointestinal stromal tumors: review on morphology, molecular pathology, prognosis, and differential diagnosis. *Arch Pathol Lab Med* 2006; **130**: 1466-1478 [PMID: 17090188]

2   **Rubin BP**, Heinrich MC, Corless CL. Gastrointestinal stromal tumour. *Lancet* 2007; **369**: 1731-1741 [PMID: 17512858 DOI: 10.1016/S0140-6736(07)60780-6]

3   **Kindblom LG**, Remotti HE, Aldenborg F, Meis-Kindblom JM. Gastrointestinal pacemaker cell tumor (GIPACT): gastrointestinal stromal tumors show phenotypic characteristics of the interstitial cells of Cajal. *Am J Pathol* 1998; **152**: 1259-1269 [PMID: 9588894]

4   **Hirota S**, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, Kawano K, Hanada M, Kurata A, Takeda M, Muhammad Tunio G, Matsuzawa Y, Kanakura Y, Shinomura Y, Kitamura Y. Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. *Science* 1998; **279**: 577-580 [PMID: 9438854 DOI: 10.1126/science.279.5350.577]

5   **Heinrich MC**, Corless CL, Duensing A, McGreevey L, Chen CJ, Joseph N, Singer S, Griffith DJ, Haley A, Town A, Demetri GD, Fletcher CD, Fletcher JA. PDGFRA activating mutations in gastrointestinal stromal tumors. *Science* 2003; **299**: 708-710 [PMID: 12522257 DOI: 10.1126/science.1079666]

6   **Miettinen M**, Sobin LH, Lasota J. Gastrointestinal stromal tumors of the stomach: a clinicopathologic, immunohistochemical, and molecular genetic study of 1765 cases with long-term follow-up. *Am J Surg Pathol* 2005; **29**: 52-68 [PMID: 15613856 DOI: 10.1097/01.pas.0000146010.92933.de]

7   **Joensuu H**. Risk stratification of patients diagnosed with gastrointestinal stromal tumor. *Hum Pathol* 2008; **39**: 1411-1419 [PMID: 18774375 DOI: 10.1016/j.humpath.2008.06.025]

8   **Nishida T**, Blay JY, Hirota S, Kitagawa Y, Kang YK. The standard diagnosis, treatment, and follow-up of gastrointestinal stromal tumors based on guidelines. *Gastric Cancer* 2016; **19**: 3-14 [PMID: 26276366 DOI: 10.1007/s10120-015-0526-8]

9   **Plaat BE**, Hollema H, Molenaar WM, Torn Broers GH, Pijpe J, Mastik MF, Hoekstra HJ, van den Berg E, Scheper RJ, van der Graaf WT. Soft tissue leiomyosarcomas and malignant gastrointestinal stromal tumors: differences in clinical outcome and expression of multidrug resistance proteins. *J Clin Oncol* 2000; **18**: 3211-3220 [PMID: 10986053 DOI: 10.1200/JCO.2000.18.18.3211]

10   **Demetri GD**, von Mehren M, Blanke CD, Van den Abbeele AD, Eisenberg B, Roberts PJ, Heinrich MC, Tuveson DA, Singer S, Janicek M, Fletcher JA, Silverman SG, Silberman SL, Capdeville R, Kiese B, Peng B, Dimitrijevic S, Druker BJ, Corless C, Fletcher CD, Joensuu H. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N Engl J Med* 2002; **347**: 472-480 [PMID: 12181401 DOI: 10.1056/NEJMoa020461]

11   **Blanke CD**, Demetri GD, von Mehren M, Heinrich MC, Eisenberg B, Fletcher JA, Corless CL, Fletcher CD, Roberts PJ, Heinz D, Wehre E, Nikolova Z, Joensuu H. Long-term results from a randomized phase II trial of standard- versus higher-dose imatinib mesylate for patients with unresectable or metastatic gastrointestinal stromal tumors expressing KIT. *J Clin Oncol* 2008; **26**: 620-625 [PMID: 18235121 DOI: 10.1200/JCO.2007.13.4403]

12   **Blanke CD**, Rankin C, Demetri GD, Ryan CW, von Mehren M, Benjamin RS, Raymond AK, Bramwell VH, Baker LH, Maki RG, Tanaka M, Hecht JR, Heinrich MC, Fletcher CD, Crowley JJ, Borden EC. Phase III randomized, intergroup trial assessing imatinib mesylate at two dose levels in patients with unresectable or metastatic gastrointestinal stromal tumors expressing the kit receptor tyrosine kinase: S0033. *J Clin Oncol* 2008; **26**: 626-632 [PMID: 18235122 DOI: 10.1200/JCO.2007.13.4452]

13   **Joensuu H**, Roberts PJ, Sarlomo-Rikala M, Andersson LC, Tervahartiala P, Tuveson D, Silberman S, Capdeville R, Dimitrijevic S, Druker B, Demetri GD. Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor. *N Engl J Med* 2001; **344**: 1052-1056 [PMID: 11287975 DOI: 10.1056/NEJM200104053441404]

14   **Verweij J**, Casali PG, Zalcberg J, LeCesne A, Reichardt P, Blay JY, Issels R, van Oosterom A, Hogendoorn PC, Van Glabbeke M, Bertulli R, Judson I. Progression-free survival in gastrointestinal stromal tumours with high-dose imatinib: randomised trial. *Lancet* 2004; **364**: 1127-1134 [PMID: 15451219 DOI: 10.1016/S0140-6736(04)17098-0]

15   **Verweij J**, van Oosterom A, Blay JY, Judson I, Rodenhuis S, van der Graaf W, Radford J, Le Cesne A, Hogendoorn PC, di Paola ED, Brown M, Nielsen OS. Imatinib mesylate (STI-571 Glivec, Gleevec) is an active agent for gastrointestinal stromal tumours, but does not yield responses in other soft-tissue sarcomas that are unselected for a molecular target. Results from an EORTC Soft Tissue and Bone Sarcoma Group phase II study. *Eur J Cancer* 2003; **39**: 2006-2011 [PMID: 12957454 DOI: 10.1016/S0959-8049(02)00836-5]

16   **van Oosterom AT**, Judson I, Verweij J, Stroobants S, Donato di Paola E, Dimitrijevic S, Martens M, Webb A, Sciot R, Van Glabbeke M, Silberman S, Nielsen OS; European Organisation for Research and Treatment of Cancer Soft Tissue and Bone Sarcoma Group. Safety and efficacy of imatinib (STI571) in metastatic gastrointestinal stromal tumours: a phase I study. *Lancet* 2001; **358**: 1421-1423 [PMID: 11705489 DOI: 10.1016/S0140-6736(01)06535-7]

17   **Choi SM**, Kim MC, Jung GJ, Kim HH, Kwon HC, Choi SR, Jang JS, Jeong JS. Laparoscopic wedge resection for gastric GIST: long-term follow-up results. *Eur J Surg Oncol* 2007; **33**: 444-447 [PMID: 17174060 DOI: 10.1016/j.ejso.2006.11.003]

18   **Sepe PS**, Brugge WR. A guide for the diagnosis and management of gastrointestinal stromal cell tumors. *Nat Rev Gastroenterol Hepatol* 2009; **6**: 363-371 [PMID: 19365407 DOI: 10.1038/nrgastro.2009.43]

19   **Faigel DO**, Abulhawa S. Gastrointestinal stromal tumors: the role of the gastroenterologist in diagnosis and risk stratification. *J Clin Gastroenterol* 2012; **46**: 629-636 [PMID: 22858511 DOI: 10.1097/MCG.0b013e3182548f6c]

20   **Mullady DK**, Tan BR. A multidisciplinary approach to the diagnosis and treatment of gastrointestinal stromal tumor. *J Clin Gastroenterol* 2013; **47**: 578-585 [PMID: 23751846 DOI: 10.1097/MCG.0b013e3182936c87]

21   **Akahoshi K**, Oya M. Gastrointestinal stromal tumor of the stomach: How to manage? *World J Gastrointest Endosc* 2010; **2**: 271-277 [PMID: 21160626 DOI: 10.4253/wjge.v2.i8.271]

22   **Corless CL**, Heinrich MC. Molecular pathobiology of gastrointestinal stromal sarcomas. *Annu Rev Pathol* 2008; **3**: 557-586 [PMID: 18039140 DOI: 10.1146/annurev.pathmechdis.3.121806.151538]

23   **Akahoshi K**, Sumida Y, Matsui N, Oya M, Akinaga R, Kubokawa M, Motomura Y, Honda K, Watanabe M, Nagaie T. Preoperative diagnosis of gastrointestinal stromal tumor by endoscopic ultrasound-guided fine needle aspiration. *World J Gastroenterol* 2007; **13**: 2077-2082 [PMID: 17465451 DOI: 10.3748/wjg.v13.i14.2077]

24   **Tran T**, Davila JA, El-Serag HB. The epidemiology of malignant gastrointestinal stromal tumors: an analysis of 1,458 cases from 1992 to 2000. *Am J Gastroenterol* 2005; **100**: 162-168 [PMID: 15654796 DOI: 10.1111/j.1572-0241.2005.40709.x]

25   **Nilsson B**, Bümming P, Meis-Kindblom JM, Odén A, Dortok A, Gustavsson B, Sablinska K, Kindblom LG. Gastrointestinal stromal tumors: the incidence, prevalence, clinical course, and prognostication in the preimatinib mesylate era--a population-based study in western Sweden. *Cancer* 2005; **103**: 821-829 [PMID: 15648083 DOI: 10.1002/cncr.20862]

26   **Tzen CY**, Wang JH, Huang YJ, Wang MN, Lin PC, Lai GL, Wu CY, Tzen CY. Incidence of gastrointestinal stromal tumor: a retrospective study based on immunohistochemical and mutational analyses. *Dig Dis Sci* 2007; **52**: 792-797 [PMID: 17253141 DOI: 10.1007/s10620-006-9480-y]

27   **Graadt van Roggen JF**, van Velthuysen ML, Hogendoorn PC. The histopathological differential diagnosis of gastrointestinal stromal tumours. *J Clin Pathol* 2001; **54**: 96-102 [PMID: 11215292 DOI: 10.1136/jcp.54.2.96]

28   **West RB**, Corless CL, Chen X, Rubin BP, Subramanian S, Montgomery K, Zhu S, Ball CA, Nielsen TO, Patel R, Goldblum JR, Brown PO, Heinrich MC, van de Rijn M. The novel marker, DOG1, is expressed ubiquitously in gastrointestinal stromal tumors irrespective of KIT or PDGFRA mutation status. *Am J Pathol* 2004; **165**: 107-113 [PMID: 15215166 DOI: 10.1016/S0002-9440(10)63279-8]

29   **Joensuu H**, Hohenberger P, Corless CL. Gastrointestinal stromal tumour. *Lancet* 2013; **382**: 973-983 [PMID: 23623056 DOI: 10.1016/S0140-6736(13)60106-3]

30   **Tryggvason G**, Kristmundsson T, Orvar K, Jónasson JG, Magnússon MK, Gíslason HG. Clinical study on gastrointestinal stromal tumors (GIST) in Iceland, 1990-2003. *Dig Dis Sci* 2007; **52**: 2249-2253 [PMID: 17420941 DOI: 10.1007/s10620-006-9248-4]

31   **Kawanowa K**, Sakuma Y, Sakurai S, Hishima T, Iwasaki Y, Saito K, Hosoya Y, Nakajima T, Funata N. High incidence of microscopic gastrointestinal stromal tumors in the stomach. *Hum Pathol* 2006; **37**: 1527-1535 [PMID: 16996566 DOI: 10.1016/j.humpath.2006.07.002]

32   **Agaimy A**, Wünsch PH, Hofstaedter F, Blaszyk H, Rümmele P, Gaumann A, Dietmaier W, Hartmann A. Minute gastric sclerosing stromal tumors (GIST tumorlets) are common in adults and frequently show c-KIT mutations. *Am J Surg Pathol* 2007; **31**: 113-120 [PMID: 17197927 DOI: 10.1097/01.pas.0000213307.05811.f0]

33   **Agaimy A**, Wünsch PH, Dirnhofer S, Bihl MP, Terracciano LM, Tornillo L. Microscopic gastrointestinal stromal tumors in esophageal and intestinal surgical resection specimens: a clinicopathologic, immunohistochemical, and molecular study of 19 lesions. *Am J Surg Pathol* 2008; **32**: 867-873 [PMID: 18408593 DOI: 10.1097/PAS.0b013e31815c0417]

34   **Abraham SC**, Krasinskas AM, Hofstetter WL, Swisher SG, Wu TT. "Seedling" mesenchymal tumors (gastrointestinal stromal tumors and leiomyomas) are common incidental tumors of the esophagogastric junction. *Am J Surg Pathol* 2007; **31**: 1629-1635 [PMID: 18059218 DOI: 10.1097/PAS.0b013e31806ab2c3]

35   **Muenst S**, Thies S, Went P, Tornillo L, Bihl MP, Dirnhofer S. Frequency, phenotype, and genotype of minute gastrointestinal stromal tumors in the stomach: an autopsy study. *Hum Pathol* 2011; **42**: 1849-1854 [PMID: 21658742 DOI: 10.1016/j.humpath.2011.01.024]

36   **Nishida T**, Kawai N, Yamaguchi S, Nishida Y. Submucosal tumors: comprehensive guide for the diagnosis and therapy of gastrointestinal submucosal tumors. *Dig Endosc* 2013; **25**: 479-489 [PMID: 23902569 DOI: 10.1111/den.12149]

37   **Hedenbro JL**, Ekelund M, Wetterberg P. Endoscopic diagnosis of submucosal gastric lesions. The results after routine endoscopy. *Surg Endosc* 1991; **5**: 20-23 [PMID: 1871670 DOI: 10.1007/BF00591381]

38   **Akahoshi K,** Inoue K, Oya M, Tamura S, Takaki M, Tatsushima S, Shiratsuchi Y, Kubokawa M, Gibo J, Yodoe K. Endoscopic ultrasonography-guided fine needle aspiration for gastrointestinal lesion *Endoscopia Digestiva* 2016; **28**: 1581-1590

39   **Akahoshi K,** Oya M, Motomura Y, Kubokawa M, Itaba S, Osoegawa T, Nakama N, Komori K, Gibo J, Yamada M, Minoda Y, Tokumaru K, Sakamoto M, Nishida K, Koga K, Nakamura K. Diagnosis of gastric submucosal tumor and submucosal tumor like lesion by endoscopic ultrasonography-guided fine needle aspiration. *Endoscopia Digestiva* 2011; **23**: 1530-1536

40   **Hwang JH**, Rulyak SD, Kimmey MB; American Gastroenterological Association Institute. American Gastroenterological Association Institute technical review on the management of gastric subepithelial masses. *Gastroenterology* 2006; **130**: 2217-2228 [PMID: 16762644 DOI: 10.1053/j.gastro.2006.04.033]

41   **Sekine M**, Imaoka H, Mizuno N, Hara K, Hijioka S, Niwa Y, Tajika M, Tanaka T, Ishihara M, Ito S, Misawa K, Ito Y, Shimizu Y, Yatabe Y, Ohnishi H, Yamao K. Clinical course of gastrointestinal stromal tumor diagnosed by endoscopic ultrasound-guided fine-needle aspiration. *Dig Endosc* 2015; **27**: 44-52 [PMID: 25059428 DOI: 10.1111/den.12333]

42   **Higuchi N**, Akahoshi K, Honda K, Matsui N, Kubokawa M, Motomura Y, Nakamura K, Takayanagi R. Diagnosis of a small splenic artery aneurysm mimicking a gastric submucosal tumor on endoscopic ultrasound. *Endoscopy* 2010; **42** Suppl 2: E107-E108 [PMID: 20306394 DOI: 10.1055/s-0029-1243940]

43   **Boyce GA**, Sivak MV Jr, Rösch T, Classen M, Fleischer DE, Boyce HW Jr, Lightdale CJ, Botet JF, Hawes RH, Lehman GA. Evaluation of submucosal upper gastrointestinal tract lesions by endoscopic ultrasound. *Gastrointest Endosc* 1991; **37**: 449-454 [PMID: 1916167 DOI: 10.1016/S0016-5107(91)70778-5]

44   **Palazzo L**, Landi B, Cellier C, Cuillerier E, Roseau G, Barbier JP. Endosonographic features predictive of benign and malignant gastrointestinal stromal cell tumours. *Gut* 2000; **46**: 88-92 [PMID: 10601061 DOI: 10.1136/gut.46.1.88]

45   **Chak A**, Canto MI, Rösch T, Dittler HJ, Hawes RH, Tio TL, Lightdale CJ, Boyce HW, Scheiman J, Carpenter SL, Van Dam J, Kochman ML, Sivak MV Jr. Endosonographic differentiation of benign and malignant stromal cell tumors. *Gastrointest Endosc* 1997; **45**: 468-473 [PMID: 9199902 DOI: 10.1016/S0016-5107(97)70175-5]

46   **Kim MN**, Kang SJ, Kim SG, Im JP, Kim JS, Jung HC, Song IS. Prediction of risk of malignancy of gastrointestinal stromal tumors by endoscopic ultrasonography. *Gut Liver* 2013; **7**: 642-647 [PMID: 24312703 DOI: 10.5009/gnl.2013.7.6.642]

47   **Hwang JH**, Saunders MD, Rulyak SJ, Shaw S, Nietsch H, Kimmey MB. A prospective study comparing endoscopy and EUS in the evaluation of GI subepithelial masses. *Gastrointest Endosc* 2005; **62**: 202-208 [PMID: 16046979 DOI: 10.1016/S0016-5107(05)01567-1]

48   **Karaca C**, Turner BG, Cizginer S, Forcione D, Brugge W. Accuracy of EUS in the evaluation of small gastric subepithelial lesions. *Gastrointest Endosc* 2010; **71**: 722-727 [PMID: 20171632 DOI: 10.1016/j.gie.2009.10.019]

49   **Kaneko E**, Kumagai J, Honda N, Nakamura S, Kino I. Evaluation of the new giant-biopsy forceps in the diagnosis of mucosal and submucosal gastric lesions. *Endoscopy* 1983; **15**: 322-326 [PMID: 6628343 DOI: 10.1055/s-2007-1021545]

50   **Scarpa M**, Bertin M, Ruffolo C, Polese L, D'Amico DF, Angriman I. A systematic review on the clinical diagnosis of gastrointestinal stromal tumors. *J Surg Oncol* 2008; **98**: 384-392 [PMID: 18668671 DOI: 10.1002/jso.21120]

51   **Cantor MJ**, Davila RE, Faigel DO. Yield of tissue sampling for subepithelial lesions evaluated by EUS: a comparison between forceps biopsies and endoscopic submucosal resection. *Gastrointest*

Endosc 2006; **64**: 29-34 [PMID: 16813799 DOI: 10.1016/j.gie.2006.02.027]

52 **Hunt GC**, Smith PP, Faigel DO. Yield of tissue sampling for submucosal lesions evaluated by EUS. *Gastrointest Endosc* 2003; **57**: 68-72 [PMID: 12518134 DOI: 10.1067/mge.2003.34]

53 **Buscaglia JM**, Nagula S, Jayaraman V, Robbins DH, Vadada D, Gross SA, DiMaio CJ, Pais S, Patel K, Sejpal DV, Kim MK. Diagnostic yield and safety of jumbo biopsy forceps in patients with subepithelial lesions of the upper and lower GI tract. *Gastrointest Endosc* 2012; **75**: 1147-1152 [PMID: 22425270 DOI: 10.1016/j.gie.2012.01.032]

54 **Mekky MA**, Yamao K, Sawaki A, Mizuno N, Hara K, Nafeh MA, Osman AM, Koshikawa T, Yatabe Y, Bhatia V. Diagnostic utility of EUS-guided FNA in patients with gastric submucosal tumors. *Gastrointest Endosc* 2010; **71**: 913-919 [PMID: 20226456 DOI: 10.1016/j.gie.2009.11.044]

55 **Larghi A**, Fuccio L, Chiarello G, Attili F, Vanella G, Paliani GB, Napoleone M, Rindi G, Larocca LM, Costamagna G, Ricci R. Fine-needle tissue acquisition from subepithelial lesions using a forward-viewing linear echoendoscope. *Endoscopy* 2014; **46**: 39-45 [PMID: 24218311 DOI: 10.1055/s-0033-1344895]

56 **Akahoshi K**, Oya M, Koga T, Koga H, Motomura Y, Kubokawa M, Gibo J, Nakamura K. Clinical usefulness of endoscopic ultrasound-guided fine needle aspiration for gastric subepithelial lesions smaller than 2 cm. *J Gastrointestin Liver Dis* 2014; **23**: 405-412 [PMID: 25531999]

57 **Hoda KM**, Rodriguez SA, Faigel DO. EUS-guided sampling of suspected GI stromal tumors. *Gastrointest Endosc* 2009; **69**: 1218-1223 [PMID: 19394006 DOI: 10.1016/j.gie.2008.09.045]

58 **Yamabe A**, Irisawa A, Bhutani MS, Shibukawa G, Abe Y, Saito A, Imbe K, Hoshi K, Igarashi R. Usefulness of endoscopic ultrasound-guided fine-needle aspiration with a forward-viewing and curved linear-array echoendoscope for small gastrointestinal subepithelial lesions. *Endosc Int Open* 2015; **3**: E161-E164 [PMID: 26135661 DOI: 10.1055/s-0034-1391671]

59 **Uesato M**, Tamachi T, Hanari N, Muto Y, Kagaya A, Urahama R, Ogura Y, Suito H, Nakano A, Aikawa M, Oide T, Matsubara H. Drill needle aspiration biopsy for submucosal tumors in an experimental study. *Gastric Cancer* 2017; **20**: 475-480 [PMID: 27530623 DOI: 10.1007/s10120-016-0630-4]

60 **Ando N**, Goto H, Niwa Y, Hirooka Y, Ohmiya N, Nagasaka T, Hayakawa T. The diagnosis of GI stromal tumors with EUS-guided fine needle aspiration with immunohistochemical analysis. *Gastrointest Endosc* 2002; **55**: 37-43 [PMID: 11756912 DOI: 10.1067/mge.2002.120323]

61 **de la Serna-Higuera C**, Pérez-Miranda M, Díez-Redondo P, Gil-Simón P, Herranz T, Pérez-Martín E, Ochoa C, Caro-Patón A. EUS-guided single-incision needle-knife biopsy: description and results of a new method for tissue sampling of subepithelial GI tumors (with video). *Gastrointest Endosc* 2011; **74**: 672-676 [PMID: 21872716 DOI: 10.1016/j.gie.2011.05.042]

62 **Mimura T**, Kuramoto S, Hashimoto M, Yamasaki K, Kobayashi K, Kobayashi M, Oohara T. Unroofing for lymphangioma of the large intestine: a new approach to endoscopic treatment. *Gastrointest Endosc* 1997; **46**: 259-263 [PMID: 9378215 DOI: 10.1016/S0016-5107(97)70097-X]

63 **Lee CK**, Chung IK, Lee SH, Lee SH, Lee TH, Park SH, Kim HS, Kim SJ, Cho HD. Endoscopic partial resection with the unroofing technique for reliable tissue diagnosis of upper GI subepithelial tumors originating from the muscularis propria on EUS (with video). *Gastrointest Endosc* 2010; **71**: 188-194 [PMID: 19879567 DOI: 10.1016/j.gie.2009.07.029]

64 **Ihara E**, Matsuzaka H, Honda K, Hata Y, Sumida Y, Akiho H, Misawa T, Toyoshima S, Chijiiwa Y, Nakamura K, Takayanagi R. Mucosal-incision assisted biopsy for suspected gastric gastrointestinal stromal tumors. *World J Gastrointest Endosc* 2013; **5**: 191-196 [PMID: 23596545 DOI: 10.4253/wjge.v5.i4.191]

65 **Dolak W**, Beer A, Kristo I, Tribl B, Asari R, Schöniger-Hekele M, Wrba F, Schoppmann SF, Trauner M, Püspök A. A retrospective study on the safety, diagnostic yield, and therapeutic effects of endoscopic unroofing for small gastric subepithelial tumors.

Gastrointest Endosc 2016; **84**: 924-929 [PMID: 27109457 DOI: 10.1016/j.gie.2016.04.019]

66 **Kobara H**, Mori H, Nishimoto N, Fujihara S, Nishiyama N, Ayaki M, Yachida T, Matsunaga T, Chiyo T, Kobayashi N, Fujita K, Kato K, Kamada H, Oryu M, Tsutsui K, Iwama H, Haba R, Masaki T. Comparison of submucosal tunneling biopsy versus EUS-guided FNA for gastric subepithelial lesions: a prospective study with crossover design. *Endosc Int Open* 2017; **5**: E695-E705 [PMID: 28782002 DOI: 10.1055/s-0043-112497]

67 **Inoue H**, Ikeda H, Hosoya T, Onimaru M, Yoshida A, Eleftheriadis N, Maselli R, Kudo S. Submucosal endoscopic tumor resection for subepithelial tumors in the esophagus and cardia. *Endoscopy* 2012; **44**: 225-230 [PMID: 22354822 DOI: 10.1055/s-0031-1291659]

68 **Ye LP**, Zhang Y, Mao XL, Zhu LH, Zhou X, Chen JY. Submucosal tunneling endoscopic resection for small upper gastrointestinal subepithelial tumors originating from the muscularis propria layer. *Surg Endosc* 2014; **28**: 524-530 [PMID: 24013472 DOI: 10.1007/s00464-013-3197-8]

69 **Standards of Practice Committee**, Faulx AL, Kothari S, Acosta RD, Agrawal D, Bruining DH, Chandrasekhara V, Eloubeidi MA, Fanelli RD, Gurudu SR, Khashab MA, Lightdale JR, Muthusamy VR, Shaukat A, Qumseya BJ, Wang A, Wani SB, Yang J, DeWitt JM. The role of endoscopy in subepithelial lesions of the GI tract. *Gastrointest Endosc* 2017; **85**: 1117-1132 [PMID: 28385194 DOI: 10.1016/j.gie.2017.02.022]

70 **Nishida T**, Hirota S, Yanagisawa A, Sugino Y, Minami M, Yamamura Y, Otani Y, Shimada Y, Takahashi F, Kubota T; GIST Guideline Subcommittee. Clinical practice guidelines for gastrointestinal stromal tumor (GIST) in Japan: English version. *Int J Clin Oncol* 2008; **13**: 416-430 [PMID: 18946752 DOI: 10.1007/s10147-008-0798-7]

71 **National Comprehensive Cancer Network**. NCCN clinical practice guidelines in oncology: soft tissue sarcoma, version 1. Available from: URL: http://www.nccn.org/professionals/physician_gls/pdf/sarcoma.pdf

72 **ESMO/European Sarcoma Network Working Group**. Gastrointestinal stromal tumours: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2014; **25** Suppl 3: iii21-iii26 [PMID: 25210085 DOI: 10.1093/annonc/mdu255]

73 **Li J**, Ye Y, Wang J, Zhang B, Qin S, Shi Y, He Y, Liang X, Liu X, Zhou Y, Wu X, Zhang X, Wang M, Gao Z, Lin T, Cao H, Shen L, Chinese Society of Clinical Oncology CSCO Expert Committee on Gastrointestinal Stromal Tumor. Chinese consensus guidelines for diagnosis and management of gastrointestinal stromal tumor. *Chin J Cancer Res* 2017; **29**: 281-293 [PMID: 28947860 DOI: 10.21147/j.issn.1000-9604.2017.04.01]

74 **Demetri GD**, von Mehren M, Antonescu CR, DeMatteo RP, Ganjoo KN, Maki RG, Pisters PW, Raut CP, Riedel RF, Schuetze S, Sundar HM, Trent JC, Wayne JD. NCCN Task Force report: update on the management of patients with gastrointestinal stromal tumors. *J Natl Compr Canc Netw* 2010; **8** Suppl 2: S1-41; quiz S42-4 [PMID: 20457867]

75 **Fong Y**, Coit DG, Woodruff JM, Brennan MF. Lymph node metastasis from soft tissue sarcoma in adults. Analysis of data from a prospective database of 1772 sarcoma patients. *Ann Surg* 1993; **217**: 72-77 [PMID: 8424704 DOI: 10.1097/00000658-199301000-00012]

76 **Koga T**, Hirayama Y, Yoshiya S, Taketani K, Nakanoko T, Yoshida R, Minagawa R, Kai M, Kajiyama K, Akahoshi K, Maehara Y. Necessity for resection of gastric gastrointestinal stromal tumors ≤ 20 mm. *Anticancer Res* 2015; **35**: 2341-2344 [PMID: 25862898]

77 **Akahoshi K**, Oya M, Koga T. Diagnosis and treatment of gastrointestinal stromal tumor (GIST). *Endoscopia Digestiva* 2012; **24**: 626-632

78 **Chen K**, Zhou YC, Mou YP, Xu XW, Jin WW, Ajoodhea H. Systematic review and meta-analysis of safety and efficacy of laparoscopic resection for gastrointestinal stromal tumors of the stomach. *Surg Endosc* 2015; **29**: 355-367 [PMID: 25005014 DOI: 10.1007/s00464-014-3676-6]

79 **Koh YX**, Chok AY, Zheng HL, Tan CS, Chow PK, Wong WK, Goh BK. A systematic review and meta-analysis comparing laparoscopic

versus open gastric resections for gastrointestinal stromal tumors of the stomach. *Ann Surg Oncol* 2013; **20**: 3549-3560 [PMID: 23793362 DOI: 10.1245/s10434-013-3051-1]

80 **Zhou PH**, Yao LQ, Qin XY, Cai MY, Xu MD, Zhong YS, Chen WF, Zhang YQ, Qin WZ, Hu JW, Liu JZ. Endoscopic full-thickness resection without laparoscopic assistance for gastric submucosal tumors originated from the muscularis propria. *Surg Endosc* 2011; **25**: 2926-2931 [PMID: 21424195 DOI: 10.1007/s00464-011-1644-y]

81 **Hiki N**, Nunobe S, Matsuda T, Hirasawa T, Yamamoto Y, Yamaguchi T. Laparoscopic endoscopic cooperative surgery. *Dig Endosc* 2015; **27**: 197-204 [PMID: 25394216 DOI: 10.1111/den.12404]

82 **Joensuu H**, Eriksson M, Sundby Hall K, Hartmann JT, Pink D, Schütte J, Ramadori G, Hohenberger P, Duyster J, Al-Batran SE, Schlemmer M, Bauer S, Wardelmann E, Sarlomo-Rikala M, Nilsson B, Sihto H, Monge OR, Bono P, Kallio R, Vehtari A, Leinonen M, Alvegård T, Reichardt P. One vs three years of adjuvant imatinib for operable gastrointestinal stromal tumor: a randomized trial. *JAMA* 2012; **307**: 1265-1272 [PMID: 22453568 DOI: 10.1001/jama.2012.347]

83 **Demetri GD**, van Oosterom AT, Garrett CR, Blackstein ME, Shah MH, Verweij J, McArthur G, Judson IR, Heinrich MC, Morgan JA, Desai J, Fletcher CD, George S, Bello CL, Huang X, Baum CM, Casali PG. Efficacy and safety of sunitinib in patients with advanced gastrointestinal stromal tumour after failure of imatinib: a randomised controlled trial. *Lancet* 2006; **368**: 1329-1338 [PMID: 17046465 DOI: 10.1016/S0140-6736(06)69446-4]

84 **Demetri GD,** Reichardt P, Kang YK, Blay JY, Rutkowski P, Gelderblom H, Hohenberger P, Leahy M, von Mehren M, Joensuu H, Badalamenti G, Blackstein M, Le Cesne A, Schöffski P, Maki RG, Bauer S, Nguyen BB, Xu J, Nishida T, Chung J, Kappeler C, Kuss I, Laurent D, Casali PG; GRID study investigators. Efficacy and safety of regorafenib for advanced gastrointestinal stromal tumours after failure of imatinib and sunitinib (GRID): an international, multicentre, randomised, placebo-controlled, phase 3 trial. *Lancet* 2013; **381**:295-302 [PMID: 23177515 DOI: 10.1016/S0140-6736(12)61857-1]

85 **Fletcher CD**, Berman JJ, Corless C, Gorstein F, Lasota J, Longley BJ, Miettinen M, O'Leary TJ, Remotti H, Rubin BP, Shmookler B, Sobin LH, Weiss SW. Diagnosis of gastrointestinal stromal tumors: A consensus approach. *Hum Pathol* 2002; **33**: 459-465 [PMID: 12094370 DOI: 10.1053/hupa.2002.123545]

86 **Miettinen M**, Lasota J. Gastrointestinal stromal tumors: pathology and prognosis at different sites. *Semin Diagn Pathol* 2006; **23**: 70-83 [PMID: 17193820 DOI: 10.1053/j.semdp.2006.09.001]

87 **Joensuu H**, Vehtari A, Riihimäki J, Nishida T, Steigen SE, Brabec P, Plank L, Nilsson B, Cirilli C, Braconi C, Bordoni A, Magnusson MK, Linke Z, Sufliarsky J, Federico M, Jonasson JG, Dei Tos AP, Rutkowski P. Risk of recurrence of gastrointestinal stromal tumour after surgery: an analysis of pooled population-based cohorts. *Lancet Oncol* 2012; **13**: 265-274 [PMID: 22153892 DOI: 10.1016/S1470-2045(11)70299-6]

88 **Joensuu H**, Martin-Broto J, Nishida T, Reichardt P, Schöffski P, Maki RG. Follow-up strategies for patients with gastrointestinal stromal tumour treated with or without adjuvant imatinib after surgery. *Eur J Cancer* 2015; **51**: 1611-1617 [PMID: 26022432 DOI: 10.1016/j.ejca.2015.05.009]

89 **Tanaka J**, Oshima T, Hori K, Tomita T, Kim Y, Watari J, Oh K, Hirota S, Matsumoto T, Miwa H. Small gastrointestinal stromal tumor of the stomach showing rapid growth and early metastasis to the liver. *Dig Endosc* 2010; **22**: 354-356 [PMID: 21175497 DOI: 10.1111/j.1443-1661.2010.01032.x]

90 **Aso A**, Ihara E, Kubo H, Osoegawa T, Oono T, Nakamura K, Ito T, Kakeji Y, Mikako O, Yamamoto H, Oishi T, Oishi Y, Hachitanda Y, Takayanagi R. Gastric gastrointestinal stromal tumor smaller than 20 mm with liver metastasis. *Clin J Gastroenterol* 2013; **6**: 29-32 [PMID: 26181401 DOI: 10.1007/s12328-012-0351-0]

91 **Gao Z,** Wang C, Xue Q, Wang J, Shen Z, Jiang K, Shen K, Liang B, Yang X, Xie Q, Wang S, Ye Y. The cut-off value of tumor size and appropriate timing of follow-up for management of minimal EUS-suspected gastric gastrointestinal stromal tumors. *BMC Gastroenterol* 2017; **17**: 8 [PMID: 28077094 DOI: 10.1186/s12876-016-0567-4]

92 **Kim MC**, Yook JH, Yang HK, Lee HJ, Sohn TS, Hyung WJ, Ryu SW, Kurokawa Y, Kim YW, Han SU, Kim HH, Park DJ, Kim W, Lee SI, Cho H, Cho GS, Kim JJ, Kim KH, Yoo MW. Long-Term Surgical Outcome of 1057 Gastric GISTs According to 7th UICC/AJCC TNM System: Multicenter Observational Study From Korea and Japan. *Medicine (Baltimore)* 2015; **94**: e1526 [PMID: 26469894 DOI: 10.1097/MD.0000000000001526]

93 **Coe TM**, Fero KE, Fanta PT, Mallory RJ, Tang CM, Murphy JD, Sicklick JK. Population-Based Epidemiology and Mortality of Small Malignant Gastrointestinal Stromal Tumors in the USA. *J Gastrointest Surg* 2016; **20**: 1132-1140 [PMID: 27025710 DOI: 10.1007/s11605-016-3134-y]

REVIEW

Biomarkers of gastric cancer: Current topics and future perspective

Tasuku Matsuoka, Masakazu Yashiro

Tasuku Matsuoak, Department of Surgical Oncology, Osaka City University Graduate School of Medicine, Osaka 545-8585, Japan

Masakazu Yashiro, Oncology Institute of Geriatrics and Medical Science, Osaka City University Graduate School of Medicine, Osaka 545-8585, Japan

ORCID number: Tasuku Matsuoka (0000-0001-5019-8519); Masakazu Yashiro (0000-0001-5743-7228).

Abstract

Gastric cancer (GC) is one of the most prevalent malignant types in the world and an aggressive disease with a poor 5-year survival. This cancer is biologically and genetically heterogeneous with a poorly understood carcinogenesis at the molecular level. Although the incidence is declining, the outcome of patients with GC remains dismal. Thus, the detection at an early stage utilizing useful screening approaches, selection of an appropriate treatment plan, and effective monitoring is pivotal to reduce GC mortalities. Identification of biomarkers in a basis of clinical information and comprehensive genome analysis could improve diagnosis, prognosis, prediction of recurrence and treatment response. This review summarized the current status and approaches in GC biomarker, which could be potentially used for early diagnosis, accurate prediction of therapeutic approaches and discussed the future perspective based on the molecular classification and profiling.

Key words: Biomarkers; Ccancer diagnosis; Prognostic marker; Predictive marker; Gastric cancer

Core tip: Gastric cancer (GC) is one of the most common leading causes of cancer death in the world. Hence, any effort in early diagnosis, choice of appropriate therapeutic strategies and efficient monitoring can have a pivotal role in reducing the disease related mortalities. Our review purpose the current trends in GC biomarker which are classified as pathologic signaling, genetic or epigenetic changes within the tumor tissue as well as non-invasive biomarkers such as blood or gastric juice based markers. These biomarkers could facilitate more individualized

treatment approaches.

INTRODUCTION

Gastric cancer (GC) is the fourth most common malignant disease and the second leading cause of cancer-related death worldwide[1]. Despite significant improvements in the survival of patients with GC over the past several decades, GC is often diagnosed at an advanced stage and prognoses is still unsatisfactory due to the high incidence of recurrence[2]. Since GC is mostly asymptomatic until it progresses to advanced stages, the early detection using effective screening approaches is important to impair GC mortalities[2]. Biomarkers are characteristics that are objectively measured and evaluated as an indicator of normal biologic process, pathogenic processes, or pharmacological response to a therapeutic intervention. Various biomarkers related to DNA, RNA, exosome, etc. have been found by recent advances in genome analysis. Development of these biomarkers in the field of cancer treatment is expected to greatly contribute to the progress of cancer, selection of appropriate therapeutic strategies and efficient follow-up programs.

GC is a heterogeneous disease in which each cancer patient exhibits a distinct genetic and molecular profile. Unfortunately, although a numerous studies has been conducted on molecular biomarkers, most of the identified biomarkers failed in the validation studies. Almost patients with advanced GC still cannot be treated with a targeted therapy and currently no diagnostic markers can be seen for secondary prevention. For being able to use GC associated biomarkers in clinical care of patients, comprehensive review to determine the direction for identifying the precise biomarker pinpoint that can be explored for the personalized therapy.

This review aims to classify developing topics for biomarkers in GC, while providing insights on potent candidates based on novel molecular classification that ultimately highlight molecular studies and clinical implementation. These findings should be useful for translating molecular classification and profiling of tumors into therapeutic targets and predictive biomarkers to achieve personalized treatment in the future.

LITERATURE SEARCH

PubMed was searched for English articles using the medical subject heading terms 'gastric cancer', and 'biomarker'. Relevant articles from clinical trials and experimental studies since 1989 were included as well as background articles relevant to the disease processes of interest. Articles which did not include biomarker analysis of GC were excluded from this review.

BIOMARKERS OF GC APLLIED IN CLINICAL PRACTICE

Gastric tumor markers have been used for the diagnosis, the determination of the clinical stage, the evaluation of treatment responses, and the screening for recurrence after successful therapy[3]. Although many biomarkers for GC including carbohydrate antigen (CA) 72-4, alpha-fetoprotein, carbohydrate antigen (CA)12-5, SLE, BCA-225, hCG and pepsinogen I/II have been reported, carcinoembryonic antigen (CEA) and CA19-9 are still the most frequently used biomarkers in clinical practice for GC.

CEA

CEA is the most widely and frequently used markers in clinical practice in the digestive tract cancer. CEA is known as an independent risk factor for predictive liver metastasis relapse[3]. Increased CEA levels are found in advanced stages of GC in a proportion of all GC patients; therefore, CEA levels are not an effective method of screening. CEA levels in peritoneal lavage fluid are said to accurately predict peritoneal recurrence after a curative resection of GC[4]. The addition of immunohistochemical CEA measurement to conventional cytology resulted in increased sensitivity. Measurement of CEA mRNA using RT-PCR is useful for detecting micrometastasis in the peritoneal cavity[5].

CA19-9

CA19-9 is a glycolipid antigen that has been identified in colorectal cancer, and it is a ligand for E-selectin, which is expressed on the surface of endothelial cells[3]. CA19-9 has previously been a commonly used marker in gastrointestinal cancer; however, it is present in a number of types of cancer, in particular pancreatic and GC. CA19-9-positive GCs demonstrated distinct clinico-pathological characteristics such as antral location, differentiated histology, prominent lymphatic and venous invasion, higher proportion of lymph node metastasis, and advanced stage[6]. Previous studies reported that the sensitivity for recurrence of CA19-9 was 56%, with a specificity of 74%[7]. Moreover, the combination of CA19-9 and other tumor markers provided more useful information for prediction of recurrence[8]. The sensitivity was reported to increase to 87% when CA19-9 was combined with CEA.

Other conventional biomarkers

Tumor markers, such as CA72-4, alpha-fetoprotein and CA125 have been widely used for the diagnosis of GC.

Although CA72-4 often represents the superior sensitivity and accuracy compared with CEA, there are few

studies on predictive screening or early detection for CA72-4 under the circumstances. AFP positive GC has the characteristics of high stage and easy occurrence to liver metastasis[9]. AFP producing GC in AFP-positive group also shows the aggressive proliferation and enhanced neovascularization compared with in AFP-negative group[10]. CA12-5 level has been said to be significantly associated with the occurrence of peritoneal dissemination in GC[3]. In patients who have carried out curative surgery, CA125 positivity may serve as the predictor of peritoneal dissemination[11].

HER2

HER2 is the first molecular biomarker available for GC patients in clinical practice. HER2, (a proto-oncogene encoded by *ERBB2* on chromosome 17) is a cell membrane surface-bound receptor tyrosine kinase and is one of the four members of the human EGFR family, including EGFR/HER1, HER2/neu, HER3, and HER4[12]. Although the significance of prognostic and predictive value of HER2 is not established in GC, the importance of HER2 as biomarker is known to be emerged. The studied HER2 amplification in patients with GC ranges from 6% to 23%[13-15]. Histological evaluation revealed the HER2 overexpression/amplification rate was predominantly seen in the intestinal-type than in diffuse-type cancers (32% *vs* 6%)[15-18].

Trastuzumab, a HER2-targeted agent, inhibits HER2-mediated signaling and prevents cleavage of the extracellular domain of HER2[13]. Trastuzumab is the first molecular targeted agent approved as standard treatment in GC. Trastuzumab for Gastric Cancer (ToGA) study, an open-label phase Ⅲ, randomized controlled trial, showed that an addition of trastuzumab to capecitabine or 5-FU and cisplatin demonstrated a clinical benefit compared to chemotherapy alone in terms of tumor response and is now considered to be the standard of care for HER2-positive GC[13]. Moreover, assessment of HER2 expression in the primary gastric tumor is a reliable foundation for examining treatment with anti-HER2 agents in patients with secondary foci[17,18]. There are several other HER2-targeted agents such as pertuzumab, lapatinib and trastuzumab emtansine being investigated in randomized clinical trials in patients with HER2-positive GC[19-21]. However, no significant evidence was found yet. Several obstacles, such as determining the suitable dose of trastuzumab, identifying a predictive biomarker, exist for the advancement of HER2-targeted therapy in GC[22]. Some researches proved the usefulness of several factors for monitoring the efficacy of trastuzumab alone or combined chemotherapy, such as p27[Kip1] and HER2-extracellular domain[23,24]. Resistance to trastuzumab is also nowadays topic in HER2 positive GCs. One of the most important mechanisms underlying trastuzumab resistance is dysregulation of phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR pathway. It is well known that *PIK3CA* mutations and phosphate and tensin homolog (PTEN) inactivation may affect the effectiveness of HER2-targeted therapy[25]. Thus, combination therapy of trastuzumab with PI3K inhibitors may provide substantial benefit in patients with HER2-positive GC. *CCNE1* amplification, one of the most popular co-occurring copy number alteration, are negatively related with the response to HER2-directed therapy, suggesting its potential role as a biomarker of resistance in patients with *ERBB2* amplified GC[26].

CURRENT TOPICS OF BIOMARKERS IN GC

The measurement of conventional serum tumor biomarkers has been widely accepted in the diagnosis and prediction of recurrence in GC. However, due to their insufficient specificity and sensitivity, these molecular markers cannot be applicated for early GC detection. Therefore, novel and dependable tumor biomarkers are urgently needed.

Metastasis related genes

FGFR2: With the progression of molecular biological techniques over the last several years, investigators have increased pivotal insights into the oncogenesis mechanisms. Besides the well-known pathogenic factor, a variety of experimental procedures have ascertained numerous oncogenes and tumor suppressor genes, including cell cycle genes in the cell growth and signaling pathways[27-29]. A well-organized clarification of these complexity of molecular and genetic profiles will lead to the precise strategies of personalized treatment. The fibroblast growth factor receptors (FGFR) family consists of four members, FGFR1, FGFR2, FGFR3 and FGFR4. These receptors bind to their high-affinity ligands, the fibroblast growth factors (FGFs)[30]. Gene amplification of FGFR induces receptor overexpression, chromosomal translocation, and point mutation or enhanced kinase activity[31]. Various basic diverse cellular behaviors and cellular processes, such as mitogenesis, differentiation, cell proliferation, angiogenesis and invasion are intermediated though FGFRs signaling pathway[30]. The frequency of overexpression of FGFR2 was 31.1% and was more common than EGFR (23.5%), HER2 (11.8%), MET (24.9%)[32]. Thus, FGFRs should attract substantial attention as a useful therapeutic candidate for targeted anticancer agents. FGFR2 amplification was found to be associated with a higher pT stage, higher pN stage, lymph node metastasis and related to poor overall survival[33]. A recent study described that FGFR expression was positively associated with the recurrent rate more than 5 years in patients with stage Ⅱ/Ⅲ GC who undergo curative surgery and adjuvant chemotherapy with S-1[34]. This result indicates that FGFR2 could be the biomarker for predicting long-term failure of adjuvant treatment of S-1 in patients with curative resection for advanced GC.

E-cadherin: E-cadherin is a transmembrane molecule

that is involved in the cellular calcium-mediated adhesion. It is encoded by *CDH1* located on the chromosome 16 (q22.1). E-cadherin closely associates to epithelial gastric cells adhesion and differentiation, which is an important prevention against the malignant formation[35]. *CDH1* is one of the most pivotal tumor suppressor genes in GC, and its disruption of activity has been proven to be closely related with the invasive and metastatic capacity[36]. The E-cadherin gene can be inactivated by several mechanisms, including *CDH1* mutations, hypermethylation, loss of heterozygosity (LOH), *H pylori* infection, transcriptional repression binding to the CDH1-E box element, and tyrosine phospholyration (*e.g.*, EGFR, MET and FGFR)[36]. Hereditary diffuse GC (HDGC) is an autosomal dominate cancer syndrome representing approximately 2% of all GCs[37]. Germline mutations in the *CDH1* gene are identified in HGDC, leading to the histological characteristics similar to diffuse-type GC. The cumulative risk of GC by 80 years of age in male *CDH1* mutation carriers is 83% for advanced GC[38]. Unfortunately, metastatic HGDC patients show lower survival compared with other sporadic GC. A recent study described that E-cadherin/catenin-EGFR crosstalk is closely associated with HDGC. Enhanced sensitivity to EGFR and PI3K kinase inhibition was induced by loss of E-cadherin/catenin-EGFR interaction in HDGC families with *CDH1* germline mutations, suggesting that these inhibitors would be an attractive tool for the targeted therapy in HGC patients in the near future.

Patients with GC showing somatic *CDH1* epigenetic and structural alterations have a worse overall survival than patients with tumors negative for *CDHI* alterations. This finding indicates that the presence of *CDH1* epigenetic and structural alterations in a diagnostic/preoperative biopsy may serve as clinically useful biomarker[39]. A recent study examined the diagnostic role of promoter methylation status of CDH1 in blood samples of patients with GC[40]. Interestingly, the significant facilitation of promoter methylation of CDH1 was shown in blood samples, suggesting that promoter methylation of CDH1 may be a good candidate of biomarkers in patients with GC.

PI3K/Akt/mTOR: PI3K/Akt/mechanistic target of rapamycin (mTOR) signaling is a crucial mediator of many essential cellular processes; genomic instability, cell cycle, growth, metabolism, survival, metastasis and resistance to chemotherapy[41]. The *PIK3CA* gene encoding the PI3K catalytic isoform p110α is the second most frequently mutated oncogene, and *PTEN* encoding the major phosphatidylisositol phosphatase is one of the most mutated tumor suppressor genes Deregulation of the PI3K/Akt/mTOR pathway can occur secondary to oncogenic mutations of *PIK3CA*[42,43]. Genetic deregulations in the PI3K/Akt/mTOR pathway have been identified frequently in GC. PI3K/Akt/mTOR expression has been associated with the lymph node status and poor survival[44]. The *PI3KCA* has been reported to be identified in 4%-25% of patients with GC[25]. Although

PIK3CA mutations have a critical role in resistance to antitumor drugs and acquisition of metastatic potential, its mutations did not likely to have an established efficient on prognosis. It has been reported that no ethnic differences in PIK3CA mutation frequencies exist, whereas the PIK3CA mutations are predominantly found in 80% of Epstein Barr virus (EBV) positive subgroups[45]. A recent study pointed that p-AKT negative tumors are more malignant than p-AKT positive but are rescued by the adjuvant chemotherapy for GC patients undergoing gastrectomy regardless of the PIK3CA mutation status[46].

MET: MET is a transmembrane tyrosine kinase receptor identified as the receptor for hepatocyte growth factor/scatter factor (HGF/SF). Activation of MET phosphorylates several signal transduction cascades, leading to cancer cell growth, angiogenesis, migration, and metastases[47]. MET amplification and/or overexpression of its secreted protein has been reported to be involved in the carcinogenesis, therapy efficacy, and outcome of GC[48,49]. The measurement and assessment of HGF activity have been crucial role in understanding the tumor microenvironment that prompt tumor metastasis and drug resistance[47]. The recent immunostaining experiment has presented that MET expression was significantly associated with lymphatic vessel invasion and poor overall survival (OS), implying that the expression of HGF/c-Met pathway might serve as a prospective predictive factor in patients with GC[50,51]. Interestingly, patients with a lower pretreatment HGF level showed a positive response to the treatment of trastuzumab. Serum level of HGF was increased in the patients who had no effect on tastuzumab compared with the pretreated level[52]. In the meanwhile, MET may be a useful predictive marker for chemotherapy, because MET signaling positively related with chemoresistance of GC therapy *via* increasing UGT1A1 level[53].

Vascular endothelial growth factor: Several signal transduction pathways are proved to be associated with tumor-associated angiogenesis, including vascular endothelial growth factor (VEGF)[54]. VEGF is a pivotal growth factor and signaling molecule to promote formation of new blood vessels. Binding to its receptor, VEGFR, activates a complex cascade of downstream signaling pathways, which leads to neovascularization, vasodilation[54]. Inhibition of VEGF and/or VEGFR activity impaired these pathways, which results in reduction of tumor proliferation, survival, and invasion. VEGF and its receptors are upregulated in 40% to 36% of cases, respectively in GC[55].

Antibodies against VEGF and VEGFR have been shown to yield anti-tumor effect, and to date, combined therapy with cytotoxic chemotherapy are adapted as standard first- or second-line treatment of GC. Ramucirumab is a recombinant humanized monoclonal antibody (mAb) specific for VEGF-R2 and impairs its activity by VEGF. Ramucirumab has provided anti-tumor effect in clinical practice as a single agent (REGARD trial) and

in combination with paclitaxel (RAINBOW trial)[56,57]. In a recent, VEGFR-2 as predictive/prognostic biomarkers has been shown in two independent phase-III studies evaluating the role of ramucirumab in GC. In the RAISE study, second-line treatment with remucirumab combined with FOLFORI presented that the group of high expression of VEGF-D had a longer survival compared with that of low expression of VEGF-D in colorectal cancer[58]. Therefore, it could be plausible that VEGF-D would be a promising predictive biomarker for ramucirumab efficacy in GC.

TP53: TP53 gene is an extremely crucial tumor suppressor which plays a role as an important regulator of different cellular processes including growth arrest and apoptosis, DNA damage, and aberrant proliferative signals[59]. The mutational site of p53 in GC is wide and the reported incidence of p53 mutations ranges from 3.2% to 65%[60]. The incidence of p53 mutation was significantly lower in EBV-GC ($n = 1$) when compared with non-EBV-GCs ($n = 10$)[61]. TP53 mutation is identified most often in the intestinal type of GC[62]. TP53 codon 72 single nucleotide polymorphism (SNP) Arg72Pro was correlated with a shorter outcome in patients with GC. TP53 codon 72 SNP was shown to predict the response to chemotherapy, and related with the time to progression in advanced GC patients treated with paclitaxel and cisplatin chemotherapy[63].

Immune checkpoint
The programmed cell death 1 (PD-1) and 2 (PD-2) are key immune checkpoint receptors expressed on activated T and B lymphocytes, natural killer T cells, and monocytes[64]. Binding of its two ligand, programmed death-1 ligands (PD-Ls) 1 and 2 to PD-1 on activated T cells leads to downregulation of cytotoxic T-cell activity and also induce immune tolerance to tumor. The expression of PD-L1 in patients with GC is ranged in 15% to 70% of cases, and they are correlated with poor outcome[65]. Targeting the PD-1 pathway and immune checkpoint blockade has proved to be a novel tool for GC treatment. Pembrolizumab and nivolumab are an anti-PD1 monoclonal antibody, and they facilitated the capacity of the immune system. A phase II study (KEYNOTE-059) demonstrated that application of pembrolizumab alone showed clinical efficacy in previously treated advanced GC[66]. Treatment of pembrolizumab showed a higher overall response rate (ORR) for patients with PD-L1 positive tumors, than in patients with PD-L1 negative tumors. Interestingly, patients with microsatellite-high (MSI-High) revealed higher response compared with in those with non-MSI-High tumors, suggesting the level of PD-L1 and MSI-High may serve as predictive biomarkers for efficacy of pembrolizumab. Besides, up-regulated expression of PD-L1/2 has been shown in the EBV-positive sub-type of tumors[67]. The results of these studies have facilitated the adaptation of immune checkpoint inhibitors generally in patients with GC.

Comprehensive gene analysis
Whole genome sequencing to targeted sequencing has played a crucial role in the identification of the genetic variations and anomalies, which leads to the development of GC. Initiation of GC is closely associated with epigenetic modifications and genome alterations. Recently, human genome project was completed and examination of gene expression profiling has been developed. Several critical genes as biomarker have been identified through genome-wide expression profile for GC[68-70]. For genome analysis, cDNA microarrays and serial analysis of gene expression (SAGE) have been mainly utilized[71]. Similar the microarray technique, SAGE is a powerful technique for worldwide analysis of gene expression in a quantitative manner without previous understanding of the gene sequences[72]. A recent cDNA microarray analysis assumed that seven genes exclusively expressed in patients with positive lymph node metastasis and five genes entirely expressed in lymph node negative patients. Genes (including *Egr-1*) which involved in cell growth, transcription and vascularization were up-regulated, whereas those in apoptosis and cell differentiation was downregulated[73]. Up-regulation of *CEACEM6, APOC1*, and *YF13H12* have been shown to be frequently up-regulated in GC[74]. In the meanwhile, significant correlation of *FUS, CDH17, COLIA1, COLIA2*, and *APOE* with invasion and metastasis was proved. A recent comprehensive analysis using SAGE and *Escherichia coli* ampicillin secretion trap (CAST) detected several gene alterations in GC. Among them, *CDH17, REG4, OLFM4, HOXA10, DSC2, TSPAN8* and *TM9SF3* were upregulated and *CLDN18* was downregulated in GC[75]. These molecules may not serve as just biomarkers but therapeutic target.

MSI
Microsatellites are repeating 1-6 nucleotide long units of DNA sequences that can be detected in both non-coding and protein coding sequences of DNA[76]. MSI is stated as somatic alterations in microsatellite sequences due to the insertion or deletion of those repeat units, which lead to genomic instability and increasing the susceptibility for the tumor development. Tumors showing 10%-29% of unstable microsatellite are considered MSI-low while tumors with $\geq$ 30% of unstable microsatellite are classified as MSI-high. In GC, 15%-30% of tumor display MSI, mainly due to epigenetic silencing thorough promoter hypermethylation of the MLH1[77]. A recent comprehensive analysis from Korea have found that more than 63% of the MSI-high GC identified the mutations within mononucleotide tracts in *TGFBR2, CEP164, MIS18BP1, RNPC3, KIAA2018, CNOT1* and *CCDC150* genes[78]. The high status of *PIK3CA* mutations in MSI positive GCs has shown the efficiency of *PIK3CA* inhibitors in the personalized treatment of MSI positive patients[79]. Studies have shown a strong association of MSI loci

in GC with intestinal type, which undergoes more genomic instability in comparison to the diffuse type[80]. Interestingly, MSI-high tumors had a better prognosis than MSI-low tumors because MSI-high tumors showed an inferior capacity of invasion and lymph node metastases[81]. A recent randomized clinical trial (MAGIC trial) reported that the prognosis of patients with MSI-high gastroesophageal cancer showed significantly longer compared with those with MSS/MSI-low when treated with surgery alone. In contrast, when patients had a treatment with surgery and perioperative chemotherapy, the prognosis was shorter in patients with MSI-high, suggesting that perioperative chemotherapy may not provide a benefit in patients with MSI-high[82]. These showing results suggest that MSI frequency may be a beneficial predictive and prognostic biomarker in patients with GC.

Epigenetic alterations

Abnormality in the epigenetic system has been caused to pathogenic mechanism, which lead to the carcinogenesis of several cancers. Numerous of research has been performed linking aberrant DNA methylation profiles and histone modifications to progressive diseases, including cancers. The most widely studied epigenetic alteration in cancer is aberrant DNA methylation[83]. In humans, DNA methylation occurs at cytosine residues that precede guanines, called CpG dinucleotides (C-phosphodiester-G). Abnormal DNA methylation in the promoter region of genes, resulted in the inactivation of tumor suppressor and other cancer-relevant genes is the most well-defined epigenetic band in GC. Various risk factors such age, chronic inflammation, and infection with *H. Pylori* and EBV can cause the aberrant gene methylation in GC[84]. Defective DNA methylation in *CDH1*, *CHFR*, *DAPK*, *GSTP1*, *p15*, *p16*, *RARβ*, *RASSF1A*, *RUNX3* and *TFPI2* has been considered as a serum biomarker for the diagnosis of GC[84,85]. Among them, the mitotic checkpoint gene, *CHFR* methylation has been found significantly elevated in mucosa from patients with GC in comparison to mucosa from normal gastric tissue. *CHFR* promoter methylation is related with tumor differentiation and lymph node involving[86]. Aberrant DNA methylation in noncancerous gastric mucosa has been implicated in gastric carcinogenesis and could be a useful biomarker for the assessing risk of GC. A recent study revealed that defect of expression of *FAT4* gene was found in highly methylated GC cell lines and impairment of methylation reduced its expression. *H. Pylori* infection has also related to methylation frequency of *FAT4* gene[87]. The understandings gained from genetic studies on molecular pathogenesis of GC may serve as the inciting cause of various experiments to identify different genetic biomarkers for early diagnosis and prognosis of this type of malignancy.

Genetic polymorphism

Genetic polymorphisms have a pivotal role in human malignancies, and the close association between cancer and genetic polymorphism for tumor initiation has been demonstrated in a variety of experimental studies[88]. One of the important genetic polymorphisms in GC is Interleukin-1β (IL1-β). IL1-β and IL-1RN have a lot of functionally related polymorphism which is associated with the secretion of IL1-β. Existence of IL-1β and IL-1RN polymorphisms with *H. pylori* infection has been shown to provide the progression of chronic atrophic gastritis and GC in an Algerian population[89]. To date, advancements of research have proved the importance of SNP in showing individual specific variations of gene aberrations. A recent study presented that the *CD44* SNP genotype, rs187116 was a meaningful prognostic factor for early recurrent GC and CD44 isoform switching from CD44v to CD44s was closely related with this effect of *CD44* rs187116 on tumor recurrence[90]. Furthermore, this *CD44* SNP was an independent risk factor for disease free survival, suggesting that *CD44* rs187116 may serve as a useful biomarker in GC patient in a Japanese population. A study to detect copy number variations and mutations found that the top mutated genes revealing high frequency were *TP53*, *SYNE1*, *CSMD3*, *LRP1B*, *CDH1*, *PIK3CA*, *ARID1A* and *PKHD*[91]. Copy number variation has been identified for *KRAS*, *JAK2*, *CD274* and *PDCD1LG2* genes using single cell resequencing amplified by different three whole genome amplification[92].

NON-INVASIVE BIOMARKERS; LIQUID BIOPSIES

The main problem to the diagnosis, treatment and surveillance of solid cancers is the necessity for getting appropriate tumor volume frequently and derived tumors does not fully represent the character of total tumor. A 'liquid biopsy' is in principle a sample of any body fluid that may contain genetic material from a tumor, for instance blood, urine, saliva or cerebrospinal fluid[93]. Progress in the field of liquid biopsies may solve the challenges with tissue biopsies by using body fluids to investigate disease biomarkers. Among the liquid biopsy options, blood samples are the most widely studied[93]. Peripheral blood samples from patients with cancer contain circulating tumor cells (CTCs), cell-free DNA, micro RNA, cell-free RNA and cell-derived vesicles, such as exosomes.

CTCs

CTCs are disseminated tumor cells as single cells or, less commonly, as cell clusters, derived from either primary tumors or metastases which are circulating in the bloodstream[94]. The existence of CTCs has been said to be clinically related with progressive or metastatic disease. Hence, CTCs can be used to monitor advanced stage disease without other surveillance markers. In particular, CTCs can be detected at an early stage before the metastasis occurs[94,95]. CTCs can thus identify patients who would have more advantage from adjuvant

treatment after surgery of primary cancer[94].

In GC, a recent meta-analysis of CTCs in patients with GC suggested associations of CTCs with prognosis, tumor staging, histologic type, and lymphovascular invasion[96]. A subset of detected CTCs with stem cell-like characteristics or epithelial-mesenchymal transition (EMT) properties, which should have the capacity for surviving and migrating to secondary foci, may play a pivotal role in tumor stage evaluation and prediction of recurrence. CD44 has been identified as a marker of GC stem cells and increased resistance for chemotherapy- or radiation-induced cell death was found in the CD44-positive GC cells[97]. The expression of epithelial markers pan-CK, E-cadherin were decreased, and mesenchymal markers N-cadherin, vimentin were overexpressed in gastric CTCs, which may provide more useful information for prediction of recurrence[98]. To date, unfortunately, utilizing CTCs in GC is not still established in clinical practice. The novel innovative approaches for detecting EMT CTCs or circulating stem cells are needed to be developed and evaluation in clinical trials should be necessary. Interestingly, a recent phase Ⅱ study presented that preselected patients whose primary tumors were HER2- but who had *HER2*+ CTCs had response rates equivalent to those reported in the trastuzumab-plus-chemotherapy arm of the ToGA study[99].

Circulating cell-free DNA

Circulating cell-free DNA (cfDNA) is cell-free extracellular DNA originating from normal and cancerous cells identicalable in the blood (the plasma or the serum)[100]. The fraction of cell‐free DNA that derived from primary tumors, metastases or from CTCs is called ctDNA. Currently, the utility of ctDNA in cancer treatment is the most extensively studied issue in cfDNA research. Compared to the restrictions of conventional biopsy which leads to significant trauma and produces small sample size, ctDNA detection displays several benefits including convenient sampling, minimal invasiveness and high repeatability. Moreover, ctDNA has been shown to be more sensitive than CTC[100]. The potential diagnostic and/or prognostic values of quantifying cf-DNA in GC patients compared to the healthy controls, have been evaluates in a variety of researches.

In GC, methylated promoter regions have been used extensively to identify ctDNA in both serum and plasma by methylation‐specific PCR. A recent meta-analysis study showed that detection of ctDNA had an obvious advantage in GC diagnosis specificity, although no superiority of ctDNA over conventional protein biomarkers was detected in sensitivity, such as CEA, CA125 and CA72-4[101]. With regard to prognostic value, significantly poorer DFS and OS in patients were identified. A recent study described that serum APC promotor 1A and RASSF1A promoter hypermethylation in *cfDNA* was a frequent epigenetic event in patients with early operable GC[102]. Interestingly, *cfDNA* showing Epstein-Barr virus (EBV) DNA has been proved to be useful for identifying the EBV-associated GC subtype, monitoring tumor development, and managing response in patients with this subtype[103]. Tumor responses to lapatinib plus Capecitabine were closely related with changes in plasma-detected *ERBB2* copy number through serial cfDNA sequencing[26].

MicroRNA

Dysregulations in non-coding regulatory RNAs can contribute to cancer initiation and development[104]. A class of small cellular RNAs, termed microRNAs (miRNAs) are 18 to 24 nucleotides noncoding RNA fragments whose function is to bind the 3'UTR region of their target gene and regulate its expression by impairing the translation[105-107]. MicroRNAs are key players in regulating several biological processes of the cell proliferation, differentiation, migration, and invasion[105].

Expression profiling of microRNAs have shown the distinctive signatures of these small regulatory RNAs in different cancers including GC[108]. Numerous microRNAs have been identified and recognized to be implicated in GC[108,109]. MiRNAs can have a critical role in cancer cell progression through EMT into metastases. The miR-200 family promotes EMT, resulting in cancer cell migration by suppressing E-cadherin and ZEB2 expressions[110]. It is known that miRNAs can increase the expression of oncogenes or reduce the expression of tumor suppressor genes[111] Abundant differentially expressed miRNAs have been associated with different stages of GC. miRNAs such as miR-21, miR-23a, miR-27a, miR-106b-25, miR-130b, miR-199a, miR-215, miR-222-221 and miR-370 were associated with oncogenic activity of GC. Whereas, miR-29a, miR-101, miR-125a, miR-129, miR-148b, miR-181c, miR-212, miR-218, miR-335, miR-375, miR-449, miR-486 and miR-512 reveal tumor suppressive activity[108].

Recently, the research for miRNA as biomarker in human malignancies has facilitated because of the unique feature of miRNAs. Cell-free miRNAs (cfmiRNAs) can be derived from cancer cells to body fluids *via* secreting exosomes particles, which lead to protected from RNase-mediated degradation in circulation, and thus are easily extractable from a variety of body fluids including blood, saliva, urine, feces *etc*. Thus, cf-miRNA could be a useful noninvasive biomarker for diagnosis and relapse of GC. Recent experimental analyses have validated expression levels of cfmiRNAs in serum are consistent with gastric tumor tissue[112]. A study based on analysis of comprehensive expression profiling of miRNAs presented that high expression of two potential biomarkers (miR-331 and miR-21) was observed in peripheral blood than in the vein draining the primary tumor and suggested as a potential diagnostic biomarker[113]. A significantly poorer OS was shown in highly miR-21 expressed group compared with low miR-21 expressed group in meta-analysis study. Several other miRNAs showed significant prognostic value in this study. Among them, miR-20b, 125a, 137, 141, 146a, 196a, 206, 218, 486-5p and 506

showed convincing as prognostic biomarkers in patients with GC[114]. Overexpression of six serum-based miRNAs (miR10b-5p, miR132-3p, miR185-5p, miR195-5p, miR-20a3p, and miR296-5p) was shown in GC compared with normal controls by using qRT-PCR-based Exiqon panel[115]. In the arm not receiving chemotherapy, high expression of miR10b-5p or miR296-5p in tissues correlated with shorter OS. Consequently, cfmiRNAs would play an increasingly important role in the diagnosis, prognosis and/or prediction of recurrence of GC. In contrast, it has been said to be difficult to utilize a miRNA as a cancer biomarker in clinical practice[116]. However, to date, clinical study are ongoing to analyze the expression level of miRNA using next generation sequencing (NGS) in GC tissue and blood by chemotherapy response (NCT03253107). Similarly, a phase II study to elucidate whether response to pralatrexate can be predicted by miR-215-5p is currently underway (NCT02050178). When these trials will complete with convincing evidence, miRNAs would be promising markers or new therapeutic targets for drug response prediction and control as well as modification of conventional adjuvant therapy.

Long noncoding RNAs

Long noncoding RNAs (lncRNAs) are sequences of nucleotides longer than 200, that can function as oncogenic or tumor-suppressor[117]. The lncRNAs act as transcriptional mediator, splicing regulator, posttranscriptional processor, enhancer, molecular sponge for miRNAs, chromatin remodeler. The lncRNAs are frequently expressed in a disease‐ or developmental‐specific manner and thus submit potential as a biomarker[111]. Nowadays over 56000 human lncRNAs populating the human genome have been identified and about 135 lncRNAs have been recognized as dysregulated in GC, so they are closely related to tumorigenesis, metastases, and prognosis[117,118]. Impaired expression of ncRuPAR significantly associated with lymph node metastasis, distant metastasis, tumor size and TNM stage in patients with GC[119]. A downregulation in the expression of AI364715, GACAT1, and GACAT2 in GC tissues could also serve as a prognostic marker[120]. LncRNA PVT1 was markedly overexpressed in GC tissues compared with that in the normal control and could be an independent prognostic marker[121,122]. However, further studies about lncRNAs are needed in order to identify their possible clinical utilization.

Exosomes

Exosomes, small cell-derived vesicles, can protect RNAs and miRNAs, from being degraded[123-127]. When exosomes were exposed to RNase the contained RNAs were protected from degradation while cellular RNA was degraded by the same RNase[126]. Exosomes hold great potential for both diagnosis and prognosis of diseases and are exceptionally useful as cancer biomarkers[128]. miR-19b and miR-106a, identified in serum-circulating exosomes, remarkably overexpressed in individuals with GC compared to healthy controls. Furthermore, the validated miRNAs were correlated to lymphatic metastasis and expressed at higher levels in stages III and IV compared to I and II stages in GC[129]. Similarly, Increased expressions of exosomal miR-21 and miR-1225-5p, isolated peritoneal lavage fluid, were exhibited in patients with T4-stage cancer compared with that in T1- to T3-stage patients[130]. These findings suggest that exosomes may serve as novel diagnostic and therapeutic biomarkers for GC.

STOMACH SPECIFIC BIOMARKER

Gastric washes/gastric juice

Because many mucosal cells can be found in stomach juice, the detection of molecular markers in stomach juice is a possible noninvasive approach to screening for GC. Gastric juice could serve as an excellent source of GC biomarkers, because these are directly released by the tumor without being excluded by the liver. Thus, gastric washes represent an alternative source for detecting aberrant DNA methylation. The analysis for the methylation levels of six genes (*ADAM23*, *GDNF*, *MINT25*, *MLF1*, *PRDM5*, *RORA*) demonstrated that a combination of the markers *MINT25*, *PRDM5* and *GDNF* achieved a high sensitivity (95%) and specificity (92%)[131]. As well, BARHL2 methylation in gastric wash DNA or gastric juice exosomal DNA significantly attenuated after endoscopic resection, suggesting that BARHL2 methylation could be useful for predicting tumor relapse[132]. The levels of PVT1 in gastric juice from gastric patients were significantly higher than those from normal subjects. PVT1 might serve as a promising biomarker for early detection and prognosis prediction of GC[121]. *Gastric juice miR-421, miR-21, miR-106a and miR-129 represent a potential biomarker for screening GC*[133].

Other specific biomarker

Micro-aerophilic, spiral-shaped Gram-negative bacterium *Helicobacter pylori (H.pylori) infection has been said to be associated with the initiation of* GC in clinico-epidemiological studies[134]. *H. pylori* Cytotoxin-associated gene A (CagA) is the first identified bacterial protein playing a positive role in the progression of GC[135]. The molecular mechanism underlying CagA-positive *H. pylori*-induced GC has been widely studied. CagA induces dysregulation of a variety of signaling pathways, including Wnt/β-catenin, PI3K/Akt, JNK, NF-κB, Hedgehog, JAK/ATAT has been identified, which results in the carcinogenesis of GC[136]. Interestingly, the development of EBV-positive GC has been shown to be prompted by *H. pylori* CagA activity, *via* SHP1 inhibition through exhibition of *PTPN6* hypermethylation[137]. In similar, *H. pylori* producing another bacterial toxin vacuolating toxin A (vacA) infection were meaningfully associated with increased risk of GC[138].

Gastrokine 1 (GKN1) is a tissue-specific 18 kDa protein that significantly expressed in gastric tissue and

Table 1 Current topics of molecular markers associated with diagnosis, prognosis, prediction of therapeutic response of gastric cancer

Marker	Alteration	Clinical purpose	Detection method	Ref.
Metastasis related genes				
Growth factors				
HER2, FGFR, PI3K/Akt/mTOR (*PIK3CA*), MET, VEGF (VEGFR-2, VEGF-D)	Overexpression	Diagnostic/prognostic/ therapeutic	Tissue	[16-18,25,32,33, 44-46,55,58]
Cell cycle regulation				
TP53	Mutation	Diagnostic	Tissue	[60,61,63]
Adhesion molecule				
E-cadherin (*CDH1*)	Mutation/epigenetic alteration	Diagnostic/prognostic	Tissue/blood	[39,40]
Immune checkpoint				
PD-L1	Mutation	Prognostic/therapeutic	Tissue	[66,67]
Comprehensive gene analysis				
CEACEM6, APOC1, YF13H12, CDH17, REG4, OLFM4, HOXA10, DSC2, TSPAN8, TM9SF3, FUS, COLIA1, COLIA2, APOE	Up-regulated	Diagnostic/prognostic/ therapeutic	Tissue	[74,75]
ATP4B, S100A9, CYP20A1, ARPC3, DDX5 CLDN18	Down-regulated	Diagnostic/prognostic/ therapeutic	Tissue	[74,75]
Microsatellite instability	High level	Prognostic/therapeutic	Tissue	[79,81,82]
Epigenetic alterations				
CDH1, CHFR, DAPK, GSTP1, p15, p16, RARβ, RASSF1A, RUNX3, TFPI2	Hypermethylation	Diagnostic	Tissue	[84-86]
Genetic polymorphism				
IL1-β, IL-1RN, CD44	SNP	Prognostic	Tissue	[89,90]
TP53, SYNE1, CSMD3, LRP1B, CDH1, PIK3CA, ARID1A, PKHD, KRAS, JAK2, CD274, PDCD1LG2	Copy number variations/ mutations	Diagnostic/prognostic/ therapeutic	Tissue	[91,92]
Circulating tumor cells				
CD44, N-cadherin, vimentin	Overexpression	Diagnostic/therapeutic	Blood	[96]
pan-CK, E-cadherin	Decreased expression	EMT process	Blood	[97]
HER2	Overexpression	Therapeutic	Blood	[99]
Circulating cell-free DNA				
APC promotor 1, RASSF1A	Hypermethylation	Diagnostic	Blood/plasma	[102]
ERBB2	Copy number variations	Therapeutic	Plasma	[26]
MicroRNA				
miR-21, miR-23a, miR-27a, miR-106b-25, miR-130b, miR-199a, miR-215, miR-222-221, miR-370	Up-regulated	Diagnostic/prognostic/ therapeutic	Blood/plasma	[108,111]
miR-29a, miR-101, miR-125a, miR-129, miR-148b, miR-181c, miR-212, miR-218, miR-335, miR-375, miR-449, miR-486, miR-512	Up-regulated	Diagnostic/prognostic/ therapeutic	Blood/plasma	[108,111]
Cell-free miRNAs				
miR-331 and miR-21	Up-regulated	Diagnostic/Prognostic	Blood	[113]
miR-20b, 125a, 137, 141, 146a, 196a, 206, 218, 486-5p	Up-regulated	Prognostic	Blood/plasma	[114]
miR10b-5p, miR132-3p, miR185-5p, miR195-5p, miR-20a3p, miR296-5p	Up-regulated	Prognostic	Plasma	[115]
Long noncoding RNAs				
ncRuPAR	Down-regulated	Diagnostic/prognostic	Tissue	[119]
AI364715, GACAT1, GACAT2	Down-regulated	Prognostic	Tissue	[120]
PVT1	Up-regulated	Prognostic	Tissue	[121]
Exosomes				
MiR-19b, miR-106a	Up-regulated	Diagnostic/prognostic	Plasma	[129]
miR-21, miR-1225-5p	Up-regulated	Diagnostic/therapeutic	PLF	[130]
Stomach specific biomarker				
ADAM23, GDNF, MINT25, MLF1, PRDM5, RORA	Hypermethylation	Diagnostic	Gastric wash	[131]
BARHL2	Hypermethylation	Diagnostic/therapeutic	Gastric wash/juice	[132]
PVT1	Up-regulated	Diagnostic/prognostic	Gastric juice	[121]
miR-421, miR-21, miR-106a, miR-129	Up-regulated	Diagnostic	Gastric juice	[133]
CagA	Up-regulated	Diagnostic	Tissue	[137]
VacA	Up-regulated	Diagnostic	Tissue	[138]
Gastrokine 1	Inactivation	Prognostic	Tissue	[139]

HER2: Human epidermal growth factor receptor 2; PLF: Peritoneal lavage fluid; FGFR: Fibroblast growth hormone receptor; PI3K: Phosphatidylinositol-3-kinase; mTOR: Mechanistic target of rapamycin; VEGF: Vascular endothelial growth factor; PD-1L: Programmed death-1 ligands; MSI: Microsatellite instability; CagA: Cytotoxin-associated gene A; VacA: Vacuolating toxin A.

is secreted into the stomach but is absent in GC. Its biological function is still unclear, but it is considered to serve as the replenishment of the surface lumen epithelial cell layer, in maintaining mucosal integrity[139]. GKN1 acts as a tumor suppressor and a modulator of apoptotic signals in GC. Due to a facilitated risk of gastric carcinogenesis in patients who have a lower expression of the protein, GKN1 could also be considered a biomarker for cancer specific to stomach. Epigenetic mechanisms leading to the inactivation of *GKN1* play a key role in the multi-step process of gastric carcinogenesis.

CONCLUSION

Through recent rapid advanced understanding of cancer biology, particularly in the field of molecular cell signaling and genetic and/or epigenetic dysregulation, the pattern of gastric carcinogenesis, and the pathways involved have become clearer. These findings may provide precious objectives for the early diagnosis of GC. Reliable prognostic and predictive markers as mentioned above may contribute to improved outcome of advanced GC. Current topics of GC biomarker based on a variety of molecular and genetic feature in this review article were summarized in Table 1. We also classified these biomarkers for early diagnosis, recurrence forecast and chemotherapy benefits assessment (Supplementary Table 1). The use of these new biomarkers such as evaluation of expression levels of various proteins and genes (*i.e.*, FGFR, CDH1, PI3K, MET, VEGFR, TP53, and PD-1) and various body fluid samples (CTC, cfDNA, miRNAs and exosomes) have opened new opportunity for diagnosis and monitoring patients with GC. And these markers will continue to be tested, developed from knowledge of novel approach, such as NGS[140]. This would facilitate more individualized treatment approaches.

FUTURE PERSPECTIVES

Although biological researchers have shown a lot of new findings in regard to biomarkers of GC to numerous publications, only conventional biomarkers (CEA, CA19-9, *etc.*) and HER2 are still in clinical use. It is urgently expected to develop biomarkers that are conventional, noninvasive, highly specific, capable of early detection and leading to treatment choice. Ideal biomarkers for early detection of cancer should be up-regulated in majority of patients with high level in cancerous tissues.

GC is a highly heterogeneous disease where even similar clinical and pathologic features lead to different outcomes, suggesting that previous staging systems may have extended to their limit of benefit for predicting patients' outcome and therapy. Thus, the novel classification of patients with GC to provide preventive and therapeutic approaches based on the genome analysis and clinical evidences are needed. In a recent, the genomic characterization of GC has led to the development of new classification by The Cancer Genome Atlas (TCGA) Research Network. The division of GC into four molecular types: (1) Tumors positive for EBV, (2) MSI-high tumors, (3) genomically stable tumors, and (4) tumors with chromosomal instability, allows identifying patients on the basis of the molecular features[67]. Future strategies aiming to translate molecular classification and profiling of tumors into therapeutic targets and predictive biomarkers in GC will be useful. The subtype of EBV-positive cancer is characterized by recurrent *PIK3CA* and *ARID1A* mutations, and high expression of PD-L1 and PD-L2, extreme DNA hypermethylation, which should be the good candidate as the diagnostic and therapeutic biomarkers. Inhibition of DNA methylation, and the suppression of immune checkpoints are promising target of this subtype. The MSI-high subtype reveals often mutation of multiple genes such as HER2 and HER3. Thus, besides the MSI, ErB family may be considerable as biomarker of this subtype. As mentioned previously, gastric MSI-high tumors represent a high frequency of PD-L1 expression. Hence, this subtype may be a pivotal candidate to anti-PD-1 therapy. The genomically stable subtype has a few somatic copy-number alterations but involves *ARID1A* and *RHOA* mutations or *CLDN18-ARHGAP* gene fusions. RhoA and its related genes could acts as the therapeutic biomarker of this subtype. The subtype with chromosomal instability is rich in TP53 mutations, and has relatively abundant amplifications of RTK genes. Therefore, this subtype can be the target therapy for RTKs, including EGFR and VEGF. The molecular classification of GC will further highlight the need for the identification and use of molecular biomarkers.

Genome wide investigation of cancer transcriptomes identified many new candidate genes. On the contrast, the candidate gene lists generated from comprehensive gene analysis vary considerably among individual studies. Therefore, it is essential to pinpoint the key players that can be explored for the development of biomarkers and leads for better cancer management. On the other hand, with regard to molecular targeting agents, their target molecules and related genes would be suitable for predicting treatment response more accurately.

The discovery of precise biomarker closely related with GC development can also be applied to treatment. We hope that this article will help design to identify the robust biomarkers in clinical care of patients and they can be relevant for the ultimate prevention and treatment of GC.

REFERENCES

1   **Jemal A**, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90 [PMID: 21296855 DOI: 10.3322/caac.20107]

2   **Wagner AD**, Unverzagt S, Grothe W, Kleber G, Grothey A, Haerting J, Fleig WE. Chemotherapy for advanced gastric cancer. *Cochrane Database Syst Rev* 2010; **3**: CD004064 [PMID: 20238327 DOI: 10.1002/14651858.CD004064.pub3]

3   **Shimada H**, Noie T, Ohashi M, Oba K, Takahashi Y. Clinical significance of serum tumor markers for gastric cancer: a systematic review of literature by the Task Force of the Japanese Gastric Cancer Association. *Gastric Cancer* 2014; **17**: 26-33 [PMID: 23572188 DOI: 10.1007/s10120-013-0259-5]

4   **Asao T**, Fukuda T, Yazawa S, Nagamachi Y. Carcinoembryonic antigen levels in peritoneal washings can predict peritoneal recurrence after curative resection of gastric cancer. *Cancer* 1991; **68**: 44-47 [PMID: 2049751 DOI: 10.1002/1097-0142(19910701)68: 1<44::AID-CNCR2820680109>3.0.CO;2-J]

5   **Zhang YS**, Xu J, Luo GH, Wang RC, Zhu J, Zhang XY, Nilsson-Ehle P, Xu N. Detection of carcinoembryonic antigen mRNA in peritoneal washes from gastric cancer patients and its clinical significance. *World J Gastroenterol* 2006; **12**: 1408-1411 [PMID: 16552810 DOI: 10.3748/wjg.v12.i9.1408]

6   **Kannagi R**, Yin J, Miyazaki K, Izawa M. Current relevance of incomplete synthesis and neo-synthesis for cancer-associated alteration of carbohydrate determinants--Hakomori's concepts revisited. *Biochim Biophys Acta* 2008; **1780**: 525-531 [PMID: 17980710 DOI: 10.1016/j.bbagen.2007.10.007]

7   **Marrelli D**, Pinto E, De Stefano A, Farnetani M, Garosi L, Roviello F. Clinical utility of CEA, CA 19-9, and CA 72-4 in the follow-up of patients with resectable gastric cancer. *Am J Surg* 2001; **181**: 16-19 [PMID: 11248169 DOI: 10.1016/S0002-9610(00)00549-3]

8   **Song YX**, Huang XZ, Gao P, Sun JX, Chen XW, Yang YC, Zhang C, Liu HP, Wang HC, Wang ZN. Clinicopathologic and Prognostic Value of Serum Carbohydrate Antigen 19-9 in Gastric Cancer: A Meta-Analysis. *Dis Markers* 2015; **2015**: 549843 [PMID: 26576068 DOI: 10.1155/2015/549843]

9   **Kono K**, Amemiya H, Sekikawa T, Iizuka H, Takahashi A, Fujii H, Matsumoto Y. Clinicopathologic features of gastric cancers producing alpha-fetoprotein. *Dig Surg* 2002; **19**: 359-365; discussion 365 [PMID: 12435906 DOI: 10.1159/000065838]

10   **Koide N**, Nishio A, Igarashi J, Kajikawa S, Adachi W, Amano J. Alpha-fetoprotein-producing gastric cancer: histochemical analysis of cell proliferation, apoptosis, and angiogenesis. *Am J Gastroenterol* 1999; **94**: 1658-1663 [PMID: 10364040 DOI: 10.1111/j.1572-0241.1999.01158.x]

11   **Namikawa T**, Kawanishi Y, Fujisawa K, Munekage E, Iwabu J, Munekage M, Maeda H, Kitagawa H, Kobayashi M, Hanazaki K. Serum carbohydrate antigen 125 is a significant prognostic marker in patients with unresectable advanced or recurrent gastric cancer. *Surg Today* 2018; **48**: 388-394 [PMID: 29043453 DOI: 10.1007/s00595-017-1598-3]

12   **Akiyama T**, Sudo C, Ogawara H, Toyoshima K, Yamamoto T. The product of the human c-erbB-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. *Science* 1986; **232**: 1644-1646 [PMID: 3012781 DOI: 10.1126/science.3012781]

13   **Bang YJ**, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Rüschoff J, Kang YK; ToGA Trial Investigators. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet* 2010; **376**: 687-697 [PMID: 20728210 DOI: 10.1016/S0140-6736(10)61121-X]

14   **Sheng WQ**, Huang D, Ying JM, Lu N, Wu HM, Liu YH, Liu JP, Bu H, Zhou XY, Du X. HER2 status in gastric cancers: a retrospective analysis from four Chinese representative clinical centers and assessment of its prognostic significance. *Ann Oncol* 2013; **24**: 2360-2364 [PMID: 23788757 DOI: 10.1093/annonc/mdt232]

15   **Gravalos C**, Jimeno A. HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target. *Ann Oncol* 2008; **19**: 1523-1529 [PMID: 18441328 DOI: 10.1093/annonc/mdn169]

16   **Moelans CB**, van Diest PJ, Milne AN, Offerhaus GJ. Her-2/neu testing and therapy in gastroesophageal adenocarcinoma. *Patholog Res Int* 2010; **2011**: 674182 [PMID: 21188213 DOI: 10.4061/2011/674182]

17   **Janjigian YY**, Werner D, Pauligk C, Steinmetz K, Kelsen DP, Jäger E, Altmannsberger HM, Robinson E, Tafe LJ, Tang LH, Shah MA, Al-Batran SE. Prognosis of metastatic gastric and gastroesophageal junction cancer by HER2 status: a European and USA International collaborative analysis. *Ann Oncol* 2012; **23**: 2656-2662 [PMID: 22689179 DOI: 10.1093/annonc/mds104]

18   **Bozzetti C**, Negri FV, Lagrasta CA, Crafa P, Bassano C, Tamagnini I, Gardini G, Nizzoli R, Leonardi F, Gasparro D, Camisa R, Cavalli S, Silini EM, Ardizzoni A. Comparison of HER2 status in primary and paired metastatic sites of gastric carcinoma. *Br J Cancer* 2011; **104**: 1372-1376 [PMID: 21487407 DOI: 10.1038/bjc.2011.121]

19   **Kang YK**, Rha SY, Tassone P, Barriuso J, Yu R, Szado T, Garg A, Bang YJ. A phase IIa dose-finding and safety study of first-line pertuzumab in combination with trastuzumab, capecitabine and cisplatin in patients with HER2-positive advanced gastric cancer. *Br J Cancer* 2014; **111**: 660-666 [PMID: 24960402 DOI: 10.1038/bjc.2014.356]

20   **Shimoyama S**. Unraveling trastuzumab and lapatinib inefficiency in gastric cancer: Future steps (Review). *Mol Clin Oncol* 2014; **2**: 175-181 [PMID: 24649329 DOI: 10.3892/mco.2013.218]

21   **Yamashita-Kashima Y**, Shu S, Harada N, Fujimoto-Ouchi K. Enhanced antitumor activity of trastuzumab emtansine (T-DM1) in combination with pertuzumab in a HER2-positive gastric cancer model. *Oncol Rep* 2013; **30**: 1087-1093 [PMID: 23783223 DOI: 10.3892/or.2013.2547]

22   **Matsuoka T**, Yashiro M. Recent advances in the HER2 targeted therapy of gastric cancer. *World J Clin Cases* 2015; **3**: 42-51 [PMID: 25610849 DOI: 10.12998/wjcc.v3.i1.42]

23   **Nahta R**, Takahashi T, Ueno NT, Hung MC, Esteva FJ. P27(kip1) down-regulation is associated with trastuzumab resistance in breast cancer cells. *Cancer Res* 2004; **64**: 3981-3986 [PMID: 15173011 DOI: 10.1158/0008-5472.CAN-03-3900]

24   **Oyama K**, Fushida S, Tsukada T, Kinoshita J, Watanabe T, Shoji M, Nakanuma S, Okamoto K, Sakai S, Makino I, Nakamura K, Hayashi H, Inokuchi M, Nakagawara H, Miyashita T, Tajima H, Takamura H, Ninomiya I, Kitagawa H, Fujimura T, Tajiri R, Ooi A, Ohta T. Evaluation of serum HER2-ECD levels in patients with gastric cancer. *J Gastroenterol* 2015; **50**: 41-45 [PMID: 24557054 DOI: 10.1007/s00535-014-0941-3]

25   **Matsuoka T**, Yashiro M. The Role of PI3K/Akt/mTOR Signaling in Gastric Carcinoma. *Cancers* (Basel) 2014; **6**: 1441-1463 [PMID: 25003395 DOI: 10.3390/cancers6031441]

26   **Kim ST**, Banks KC, Pectasides E, Kim SY, Kim K, Lanman RB, Talasaz A, An J, Choi MG, Lee JH, Sohn TS, Bae JM, Kim S, Park SH, Park JO, Park YS, Lim HY, Kim NKD, Park W, Lee H, Bass AJ, Kim K, Kang WK, Lee J. Impact of genomic alterations on lapatinib treatment outcome and cell-free genomic landscape during HER2 therapy in HER2+ gastric cancer patients. *Ann Oncol* 2018; **29**: 1037-1048 [PMID: 29409051 DOI: 10.1093/annonc/mdy034]

27   **Hamashima C**, Shibuya D, Yamazaki H, Inoue K, Fukao A, Saito H, Sobue T. The Japanese guidelines for gastric cancer screening. *Jpn J Clin Oncol* 2008; **38**: 259-267 [PMID: 18344316 DOI: 10.1093/jjco/hyn017]

28   **Nobili S**, Bruno L, Landini I, Napoli C, Bechi P, Tonelli F, Rubio CA, Mini E, Nesi G. Genomic and genetic alterations influence the progression of gastric cancer. *World J Gastroenterol* 2011; **17**: 290-299 [PMID: 21253387 DOI: 10.3748/wjg.v17.i3.290]

29   **Leung WK**, Wu MS, Kakugawa Y, Kim JJ, Yeoh KG, Goh KL, Wu KC, Wu DC, Sollano J, Kachintorn U, Gotoda T, Lin JT, You WC, Ng EK, Sung JJ; Asia Pacific Working Group on Gastric Cancer. Screening for gastric cancer in Asia: current evidence and practice. *Lancet Oncol* 2008; **9**: 279-287 [PMID: 18308253 DOI: 10.1016/S1470-2045(08)70072-X]

30   **Yashiro M**, Matsuoka T. Fibroblast growth factor receptor signaling as therapeutic targets in gastric cancer. *World J Gastroenterol* 2016; **22**: 2415-2423 [PMID: 26937130 DOI: 10.3748/wjg.v22.i8.2415]

31   **Greulich H**, Pollock PM. Targeting mutant fibroblast growth factor receptors in cancer. *Trends Mol Med* 2011; **17**: 283-292 [PMID: 21367659 DOI: 10.1016/j.molmed.2011.01.012]

32   **Nagatsuma AK**, Aizawa M, Kuwata T, Doi T, Ohtsu A, Fujii H, Ochiai A. Expression profiles of HER2, EGFR, MET and FGFR2 in a large cohort of patients with gastric adenocarcinoma. *Gastric Cancer* 2015; **18**: 227-238 [PMID: 24626858 DOI: 10.1007/s10120-014-0360-4]

33   **Betts G**, Valentine H, Pritchard S, Swindell R, Williams V, Morgan S, Griffiths EA, Welch I, West C, Womack C. FGFR2, HER2 and cMet in gastric adenocarcinoma: detection, prognostic significance and

assessment of downstream pathway activation. *Virchows Arch* 2014; **464**: 145-156 [PMID: 24306956 DOI: 10.1007/s00428-013-1517-y]

34   **Hosoda K**, Yamashita K, Ushiku H, Ema A, Moriya H, Mieno H, Washio M, Watanabe M. Prognostic relevance of FGFR2 expression in stage Ⅱ/Ⅲ gastric cancer with curative resection and S-1 chemotherapy. *Oncol Lett* 2018; **15**: 1853-1860 [PMID: 29434882 DOI: 10.3892/ol.2017.7515]

35   **Carneiro P**, Fernandes MS, Figueiredo J, Caldeira J, Carvalho J, Pinheiro H, Leite M, Melo S, Oliveira P, Simões-Correia J, Oliveira MJ, Carneiro F, Figueiredo C, Paredes J, Oliveira C, Seruca R. E-cadherin dysfunction in gastric cancer--cellular consequences, clinical applications and open questions. *FEBS Lett* 2012; **586**: 2981-2989 [PMID: 22841718 DOI: 10.1016/j.febslet.2012.07.045]

36   **Chan AO**. E-cadherin in gastric cancer. *World J Gastroenterol* 2006; **12**: 199-203 [PMID: 16482618 DOI: 10.3748/wjg.v12.i2.199]

37   **Lynch HT**, Kaurah P, Wirtzfeld D, Rubinstein WS, Weissman S, Lynch JF, Grady W, Wiyrick S, Senz J, Huntsman DG. Hereditary diffuse gastric cancer: diagnosis, genetic counseling, and prophylactic total gastrectomy. *Cancer* 2008; **112**: 2655-2663 [PMID: 18442100 DOI: 10.1002/cncr.23501]

38   **Pharoah PD**, Guilford P, Caldas C; International Gastric Cancer Linkage Consortium. Incidence of gastric cancer and breast cancer in CDH1 (E-cadherin) mutation carriers from hereditary diffuse gastric cancer families. *Gastroenterology* 2001; **121**: 1348-1353 [PMID: 11729114 DOI: 10.1053/gast.2001.29611]

39   **Corso G**, Carvalho J, Marrelli D, Vindigni C, Carvalho B, Seruca R, Roviello F, Oliveira C. Somatic mutations and deletions of the E-cadherin gene predict poor survival of patients with gastric cancer. *J Clin Oncol* 2013; **31**: 868-875 [PMID: 23341533 DOI: 10.1200/JCO.2012.44.4612]

40   **Wen J**, Zheng T, Hu K, Zhu C, Guo L, Ye G. Promoter methylation of tumor-related genes as a potential biomarker using blood samples for gastric cancer detection. *Oncotarget* 2017; **8**: 77783-77793 [PMID: 29100425 DOI: 10.18632/oncotarget.20782]

41   **Hanahan D**, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]

42   **Samuels Y**, Ericson K. Oncogenic PI3K and its role in cancer. *Curr Opin Oncol* 2006; **18**: 77-82 [PMID: 16357568 DOI: 10.1097/01.cco.0000198021.99347.b9]

43   **Song MS**, Salmena L, Pandolfi PP. The functions and regulation of the PTEN tumour suppressor. *Nat Rev Mol Cell Biol* 2012; **13**: 283-296 [PMID: 22473468 DOI: 10.1038/nrm3330]

44   **Xu DZ**, Geng QR, Tian Y, Cai MY, Fang XJ, Zhan YQ, Zhou ZW, Li W, Chen YB, Sun XW, Guan YX, Li YF, Lin TY. Activated mammalian target of rapamycin is a potential therapeutic target in gastric cancer. *BMC Cancer* 2010; **10**: 536 [PMID: 20929525 DOI: 10.1186/1471-2407-10-536]

45   **Iizasa H**, Nanbo A, Nishikawa J, Jinushi M, Yoshiyama H. Epstein-Barr Virus (EBV)-associated gastric carcinoma. *Viruses* 2012; **4**: 3420-3439 [PMID: 23342366 DOI: 10.3390/v4123420]

46   **Ito C**, Nishizuka SS, Ishida K, Uesugi N, Sugai T, Tamura G, Koeda K, Sasaki A. Analysis of PIK3CA mutations and PI3K pathway proteins in advanced gastric cancer. *J Surg Res* 2017; **212**: 195-204 [PMID: 28550907 DOI: 10.1016/j.jss.2017.01.018]

47   **Matsumoto K**, Umitsu M, De Silva DM, Roy A, Bottaro DP. Hepatocyte growth factor/MET in cancer progression and biomarker discovery. *Cancer Sci* 2017; **108**: 296-307 [PMID: 28064454 DOI: 10.1111/cas.13156]

48   **Graziano F**, Arduini F, Ruzzo A, Bearzi I, Humar B, More H, Silva R, Muretto P, Guilford P, Testa E, Mari D, Magnani M, Cascinu S. Prognostic analysis of E-cadherin gene promoter hypermethylation in patients with surgically resected, node-positive, diffuse gastric cancer. *Clin Cancer Res* 2004; **10**: 2784-2789 [PMID: 15102685 DOI: 10.1158/1078-0432.CCR-03-0320]

49   **Lee HE**, Kim MA, Lee HS, Jung EJ, Yang HK, Lee BL, Bang YJ, Kim WH. MET in gastric carcinomas: comparison between protein expression and gene copy number and impact on clinical outcome. *Br J Cancer* 2012; **107**: 325-333 [PMID: 22644302 DOI: 10.1038/bjc.2012.237]

50   **Noguchi E**, Saito N, Kobayashi M, Kameoka S. Clinical significance of hepatocyte growth factor/c-Met expression in the assessment of gastric cancer progression. *Mol Med Rep* 2015; **11**: 3423-3431 [PMID: 25592281 DOI: 10.3892/mmr.2015.3205]

51   **Huang X**, Wang C, Sun J, Luo J, You J, Liao L, Li M. Clinical value of CagA, c-Met, PI3K and Beclin-1 expressed in gastric cancer and their association with prognosis. *Oncol Lett* 2018; **15**: 947-955 [PMID: 29422968 DOI: 10.3892/ol.2017.7394]

52   **Takahashi N**, Furuta K, Taniguchi H, Sasaki Y, Shoji H, Honma Y, Iwasa S, Okita N, Takashima A, Kato K, Hamaguchi T, Shimada Y, Yamada Y. Serum level of hepatocyte growth factor is a novel marker of predicting the outcome and resistance to the treatment with trastuzumab in HER2-positive patients with metastatic gastric cancer. *Oncotarget* 2016; **7**: 4925-4938 [PMID: 26716644 DOI: 10.18632/oncotarget.6753]

53   **Yashiro M**, Nishii T, Hasegawa T, Matsuzaki T, Morisaki T, Fukuoka T, Hirakawa K. A c-Met inhibitor increases the chemosensitivity of cancer stem cells to the irinotecan in gastric carcinoma. *Br J Cancer* 2013; **109**: 2619-2628 [PMID: 24129235 DOI: 10.1038/bjc.2013.638]

54   **Ylä-Herttuala S**, Rissanen TT, Vajanto I, Hartikainen J. Vascular endothelial growth factors: biology and current status of clinical applications in cardiovascular medicine. *J Am Coll Cardiol* 2007; **49**: 1015-1026 [PMID: 17349880 DOI: 10.1016/j.jacc.2006.09.053]

55   **Lieto E**, Ferraraccio F, Orditura M, Castellano P, Mura AL, Pinto M, Zamboli A, De Vita F, Galizia G. Expression of vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) is an independent prognostic indicator of worse outcome in gastric cancer patients. *Ann Surg Oncol* 2008; **15**: 69-79 [PMID: 17896140 DOI: 10.1245/s10434-007-9596-0]

56   **Fuchs CS**, Tomasek J, Yong CJ, Dumitru F, Passalacqua R, Goswami C, Safran H, Dos Santos LV, Aprile G, Ferry DR, Melichar B, Tehfe M, Topuzov E, Zalcberg JR, Chau I, Campbell W, Sivanandan C, Pikiel J, Koshiji M, Hsu Y, Liepa AM, Gao L, Schwartz JD, Tabernero J; REGARD Trial Investigators. Ramucirumab monotherapy for previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (REGARD): an international, randomised, multicentre, placebo-controlled, phase 3 trial. *Lancet* 2014; **383**: 31-39 [PMID: 24094768 DOI: 10.1016/S0140-6736(13)61719-5]

57   **Wilke H**, Muro K, Van Cutsem E, Oh SC, Bodoky G, Shimada Y, Hironaka S, Sugimoto N, Lipatov O, Kim TY, Cunningham D, Rougier P, Komatsu Y, Ajani J, Emig M, Carlesi R, Ferry D, Chandrawansa K, Schwartz JD, Ohtsu A; RAINBOW Study Group. Ramucirumab plus paclitaxel versus placebo plus paclitaxel in patients with previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (RAINBOW): a double-blind, randomised phase 3 trial. *Lancet Oncol* 2014; **15**: 1224-1235 [PMID: 25240821 DOI: 10.1016/S1470-2045(14)70420-6]

58   **Tabernero J**, Hozak RR, Yoshino T, Cohn AL, Obermannova R, Bodoky G, Garcia-Carbonero R, Ciuleanu TE, Portnoy DC, Prausová J, Muro K, Siegel RW, Konrad RJ, Ouyang H, Melemed SA, Ferry D, Nasroulah F, Van Cutsem E. Analysis of angiogenesis biomarkers for ramucirumab efficacy in patients with metastatic colorectal cancer from RAISE, a global, randomized, double-blind, phase Ⅲ study. *Ann Oncol* 2018; **29**: 602-609 [PMID: 29228087 DOI: 10.1093/annonc/mdx767]

59   **Levine AJ**. p53, the cellular gatekeeper for growth and division. *Cell* 1997; **88**: 323-331 [PMID: 9039259 DOI: 10.1016/S0092-8674(00)81871-1]

60   **Oki E**, Zhao Y, Yoshida R, Egashira A, Ohgaki K, Morita M, Kakeji Y, Maehara Y. The difference in p53 mutations between cancers of the upper and lower gastrointestinal tract. *Digestion* 2009; **79** Suppl 1: 33-39 [PMID: 19153488 DOI: 10.1159/000167864]

61   **Lee J**, van Hummelen P, Go C, Palescandolo E, Jang J, Park HY, Kang SY, Park JO, Kang WK, MacConaill L, Kim KM. High-throughput mutation profiling identifies frequent somatic mutations in advanced gastric adenocarcinoma. *PLoS One* 2012; **7**: e38892 [PMID: 22723903 DOI: 10.1371/journal.pone.0038892]

62   **Endoh Y**, Sakata K, Tamura G, Ohmura K, Ajioka Y, Watanabe H, Motoyama T. Cellular phenotypes of differentiated-type adenocarcinomas and precancerous lesions of the stomach are

dependent on the genetic pathways. *J Pathol* 2000; **191**: 257-263 [PMID: 10878546 DOI: 10.1002/1096-9896(2000)9999:9999<:: AID-PATH631>3.0.CO;2-2]

63   **Li QF**, Yao RY, Liu KW, Lv HY, Jiang T, Liang J. Genetic polymorphism of GSTP1: prediction of clinical outcome to oxaliplatin/5-FU-based chemotherapy in advanced gastric cancer. *J Korean Med Sci* 2010; **25**: 846-852 [PMID: 20514304 DOI: 10.3346/jkms.2010.25.6.846]

64   **Sharpe AH**, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol* 2007; **8**: 239-245 [PMID: 17304234 DOI: 10.1038/ni1443]

65   **Gu L**, Chen M, Guo D, Zhu H, Zhang W, Pan J, Zhong X, Li X, Qian H, Wang X. PD-L1 and gastric cancer prognosis: A systematic review and meta-analysis. *PLoS One* 2017; **12**: e0182692 [PMID: 28796808 DOI: 10.1371/journal.pone.0182692]

66   **Curea FG**, Hebbar M, Ilie SM, Bacinschi XE, Trifanescu OG, Botnariuc I, Anghel RM. Current Targeted Therapies in HER2-Positive Gastric Adenocarcinoma. *Cancer Biother Radiopharm* 2017; **32**: 351-363 [PMID: 29265917 DOI: 10.1089/cbr.2017.2249]

67   **Cancer Genome Atlas Research Network**. Comprehensive molecular characterization of gastric adenocarcinoma. *Nature* 2014; **513**: 202-209 [PMID: 25079317 DOI: 10.1038/nature13480]

68   **Cho JY**, Lim JY, Cheong JH, Park YY, Yoon SL, Kim SM, Kim SB, Kim H, Hong SW, Park YN, Noh SH, Park ES, Chu IS, Hong WK, Ajani JA, Lee JS. Gene expression signature-based prognostic risk score in gastric cancer. *Clin Cancer Res* 2011; **17**: 1850-1857 [PMID: 21447720 DOI: 10.1158/1078-0432.CCR-10-2180]

69   **Cui J**, Chen Y, Chou WC, Sun L, Chen L, Suo J, Ni Z, Zhang M, Kong X, Hoffman LL, Kang J, Su Y, Olman V, Johnson D, Tench DW, Amster IJ, Orlando R, Puett D, Li F, Xu Y. An integrated transcriptomic and computational analysis for biomarker identification in gastric cancer. *Nucleic Acids Res* 2011; **39**: 1197-1207 [PMID: 20965966 DOI: 10.1093/nar/gkq960]

70   **Hippo Y**, Taniguchi H, Tsutsumi S, Machida N, Chong JM, Fukayama M, Kodama T, Aburatani H. Global gene expression analysis of gastric cancer by oligonucleotide microarrays. *Cancer Res* 2002; **62**: 233-240 [PMID: 11782383]

71   **DeRisi J**, Penland L, Brown PO, Bittner ML, Meltzer PS, Ray M, Chen Y, Su YA, Trent JM. Use of a cDNA microarray to analyse gene expression patterns in human cancer. *Nat Genet* 1996; **14**: 457-460 [PMID: 8944026 DOI: 10.1038/ng1296-457]

72   **Velculescu VE**, Zhang L, Vogelstein B, Kinzler KW. Serial analysis of gene expression. *Science* 1995; **270**: 484-487 [PMID: 7570003 DOI: 10.1126/science.270.5235.484]

73   **Ojetti V**, Persiani R, Cananzi FC, Sensi C, Piscaglia AC, Saulnier N, Biondi A, Gasbarrini A, D'Ugo D. cDNA-microarray analysis as a new tool to predict lymph node metastasis in gastric cancer. *World J Surg* 2014; **38**: 2058-2064 [PMID: 24696059 DOI: 10.1007/s00268-014-2529-8]

74   **Yasui W**, Oue N, Ito R, Kuraoka K, Nakayama H. Search for new biomarkers of gastric cancer through serial analysis of gene expression and its clinical implications. *Cancer Sci* 2004; **95**: 385-392 [PMID: 15132764 DOI: 10.1111/j.1349-7006.2004.tb03220.x]

75   **Oue N**, Sentani K, Sakamoto N, Yasui W. Clinicopathologic and molecular characteristics of gastric cancer showing gastric and intestinal mucin phenotype. *Cancer Sci* 2015; **106**: 951-958 [PMID: 26033320 DOI: 10.1111/cas.12706]

76   **Aaltonen LA**, Peltomäki P, Leach FS, Sistonen P, Pylkkänen L, Mecklin JP, Järvinen H, Powell SM, Jen J, Hamilton SR. Clues to the pathogenesis of familial colorectal cancer. *Science* 1993; **260**: 812-816 [PMID: 8484121 DOI: 10.1126/science.8484121]

77   **Pinto M**, Oliveira C, Machado JC, Cirnes L, Tavares J, Carneiro F, Hamelin R, Hofstra R, Seruca R, Sobrinho-Simões M. MSI-L gastric carcinomas share the hMLH1 methylation status of MSI-H carcinomas but not their clinicopathological profile. *Lab Invest* 2000; **80**: 1915-1923 [PMID: 11140703 DOI: 10.1038/labinvest.3780201]

78   **Yoon K**, Lee S, Han TS, Moon SY, Yun SM, Kong SH, Jho S, Choe J, Yu J, Lee HJ, Park JH, Kim HM, Lee SY, Park J, Kim WH, Bhak J, Yang HK, Kim SJ. Comprehensive genome- and transcriptome-wide analyses of mutations associated with microsatellite instability in Korean gastric cancers. *Genome Res* 2013; **23**: 1109-1117 [PMID: 23737375 DOI: 10.1101/gr.145706.112]

79   **Zang ZJ**, Cutcutache I, Poon SL, Zhang SL, McPherson JR, Tao J, Rajasegaran V, Heng HL, Deng N, Gan A, Lim KH, Ong CK, Huang D, Chin SY, Tan IB, Ng CC, Yu W, Wu Y, Lee M, Wu J, Poh D, Wan WK, Rha SY, So J, Salto-Tellez M, Yeoh KG, Wong WK, Zhu YJ, Futreal PA, Pang B, Ruan Y, Hillmer AM, Bertrand D, Nagarajan N, Rozen S, Teh BT, Tan P. Exome sequencing of gastric adenocarcinoma identifies recurrent somatic mutations in cell adhesion and chromatin remodeling genes. *Nat Genet* 2012; **44**: 570-574 [PMID: 22484628 DOI: 10.1038/ng.2246]

80   **Shokal U**, Sharma PC. Implication of microsatellite instability in human gastric cancers. *Indian J Med Res* 2012; **135**: 599-613 [PMID: 22771588]

81   **dos Santos NR**, Seruca R, Constância M, Seixas M, Sobrinho-Simões M. Microsatellite instability at multiple loci in gastric carcinoma: clinicopathologic implications and prognosis. *Gastroenterology* 1996; **110**: 38-44 [PMID: 8536886 DOI: 10.1053/gast.1996.v110.pm8536886]

82   **Smyth EC**, Wotherspoon A, Peckitt C, Gonzalez D, Hulkki-Wilson S, Eltahir Z, Fassan M, Rugge M, Valeri N, Okines A, Hewish M, Allum W, Stenning S, Nankivell M, Langley R, Cunningham D. Mismatch Repair Deficiency, Microsatellite Instability, and Survival: An Exploratory Analysis of the Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) Trial. *JAMA Oncol* 2017; **3**: 1197-1203 [PMID: 28241187 DOI: 10.1001/jamaoncol.2016.6762]

83   **Kim H**, Wang X, Jin P. Developing DNA methylation-based diagnostic biomarkers. *J Genet Genomics* 2018; **45**: 87-97 [PMID: 29496486 DOI: 10.1016/j.jgg.2018.02.003]

84   **Qu Y**, Dang S, Hou P. Gene methylation in gastric cancer. *Clin Chim Acta* 2013; **424**: 53-65 [PMID: 23669186 DOI: 10.1016/j.cca.2013.05.002]

85   **Sapari NS**, Loh M, Vaithilingam A, Soong R. Clinical potential of DNA methylation in gastric cancer: a meta-analysis. *PLoS One* 2012; **7**: e36275 [PMID: 22558417 DOI: 10.1371/journal.pone.0036275]

86   **Ding Y**, Lian HF, Du Y. Clinicopathological significance of CHFR promoter methylation in gastric cancer: a meta-analysis. *Oncotarget* 2017; **9**: 10083-10090 [PMID: 29515792 DOI: 10.18632/oncotarget.23394]

87   **Yoshida S**, Yamashita S, Niwa T, Mori A, Ito S, Ichinose M, Ushijima T. Epigenetic inactivation of FAT4 contributes to gastric field cancerization. *Gastric Cancer* 2017; **20**: 136-145 [PMID: 26792292 DOI: 10.1007/s10120-016-0593-5]

88   **Dixon K**, Kopras E. Genetic alterations and DNA repair in human carcinogenesis. *Semin Cancer Biol* 2004; **14**: 441-448 [PMID: 15489137 DOI: 10.1016/j.semcancer.2004.06.007]

89   **Drici Ael-M**, Moulessehoul S, Tifrit A, Diaf M, Turki DK, Bachir M, Tou A. Effect of IL-1β and IL-1RN polymorphisms in carcinogenesis of the gastric mucosa in patients infected with Helicobacter pylori in Algeria. *Libyan J Med* 2016; **11**: 31576 [PMID: 27340011 DOI: 10.3402/ljm.v11.31576]

90   **Suenaga M**, Yamada S, Fuchs BC, Fujii T, Kanda M, Tanaka C, Kobayashi D, Fujiwara M, Tanabe KK, Kodera Y. CD44 single nucleotide polymorphism and isoform switching may predict gastric cancer recurrence. *J Surg Oncol* 2015; **112**: 622-628 [PMID: 26416034 DOI: 10.1002/jso.24056]

91   **Kuboki Y**, Yamashita S, Niwa T, Ushijima T, Nagatsuma A, Kuwata T, Yoshino T, Doi T, Ochiai A, Ohtsu A. Comprehensive analyses using next-generation sequencing and immunohistochemistry enable precise treatment in advanced gastric cancer. *Ann Oncol* 2016; **27**: 127-133 [PMID: 26489445 DOI: 10.1093/annonc/mdv508]

92   **Hou Y**, Wu K, Shi X, Li F, Song L, Wu H, Dean M, Li G, Tsang S, Jiang R, Zhang X, Li B, Liu G, Bedekar N, Lu N, Xie G, Liang H, Chang L, Wang T, Chen J, Li Y, Zhang X, Yang H, Xu X, Wang L, Wang J. Comparison of variations detection between whole-genome amplification methods used in single-cell resequencing. *Gigascience* 2015; **4**: 37 [PMID: 26251698 DOI: 10.1186/s13742-015-0068-3]

93   **Siravegna G**, Marsoni S, Siena S, Bardelli A. Integrating liquid biopsies into the management of cancer. *Nat Rev Clin Oncol* 2017; **14**: 531-548 [PMID: 28252003 DOI: 10.1038/nrclinonc.2017.14]

94   **Allard WJ**, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, Tibbe AG, Uhr JW, Terstappen LW. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin Cancer Res* 2004; **10**: 6897-6904 [PMID: 15501967 DOI: 10.1158/1078-0432.CCR-04-0378]

95   **Glaves D**. Correlation between circulating cancer cells and incidence of metastases. *Br J Cancer* 1983; **48**: 665-673 [PMID: 6639858 DOI: 10.1038/bjc.1983.248]

96   **Huang X**, Gao P, Sun J, Chen X, Song Y, Zhao J, Xu H, Wang Z. Clinicopathological and prognostic significance of circulating tumor cells in patients with gastric cancer: a meta-analysis. *Int J Cancer* 2015; **136**: 21-33 [PMID: 24803400 DOI: 10.1002/ijc.28954]

97   **Takaishi S**, Okumura T, Tu S, Wang SS, Shibata W, Vigneshwaran R, Gordon SA, Shimada Y, Wang TC. Identification of gastric cancer stem cells using the cell surface marker CD44. *Stem Cells* 2009; **27**: 1006-1020 [PMID: 19415765 DOI: 10.1002/stem.30]

98   **Yuan D**, Xia H, Zhang Y, Chen L, Leng W, Chen T, Chen Q, Tang Q, Mo X, Liu M, Bi F. P-Akt/miR-200 signaling regulates epithelial-mesenchymal transition, migration and invasion in circulating gastric tumor cells. *Int J Oncol* 2014; **45**: 2430-2438 [PMID: 25200917 DOI: 10.3892/ijo.2014.2644]

99   **Mishima Y**, Matsusaka S, Chin K, Mikuniya M, Minowa S, Takayama T, Shibata H, Kuniyoshi R, Ogura M, Terui Y, Mizunuma N, Hatake K. Detection of HER2 Amplification in Circulating Tumor Cells of HER2-Negative Gastric Cancer Patients. *Target Oncol* 2017; **12**: 341-351 [PMID: 28508152 DOI: 10.1007/s11523-017-0493-6]

100   **Qi Q**, Pan YF, Shen JJ, Gu XQ, Han SW, Liao HH, Jiang YZ, Zhong LP. Circulating DNA for detection of gastric cancer. *Eur Rev Med Pharmacol Sci* 2016; **20**: 2558-2564 [PMID: 27383305]

101   **Gao Y**, Zhang K, Xi H, Cai A, Wu X, Cui J, Li J, Qiao Z, Wei B, Chen L. Diagnostic and prognostic value of circulating tumor DNA in gastric cancer: a meta-analysis. *Oncotarget* 2017; **8**: 6330-6340 [PMID: 28009985 DOI: 10.18632/oncotarget.14064]

102   **Balgkouranidou I**, Matthaios D, Karayiannakis A, Bolanaki H, Michailidis P, Xenidis N, Amarantidis K, Chelis L, Trypsianis G, Chatzaki E, Lianidou ES, Kakolyris S. Prognostic role of APC and RASSF1A promoter methylation status in cell free circulating DNA of operable gastric cancer patients. *Mutat Res* 2015; **778**: 46-51 [PMID: 26073472 DOI: 10.1016/j.mrfmmm.2015.05.002]

103   **Shoda K**, Ichikawa D, Fujita Y, Masuda K, Hiramoto H, Hamada J, Arita T, Konishi H, Kosuga T, Komatsu S, Shiozaki A, Okamoto K, Imoto I, Otsuji E. Clinical utility of circulating cell-free Epstein-Barr virus DNA in patients with gastric cancer. *Oncotarget* 2017; **8**: 28796-28804 [PMID: 28430637 DOI: 10.18632/oncotarget.15675]

104   **Calin GA**, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006; **6**: 857-866 [PMID: 17060945 DOI: 10.1038/nrc1997]

105   **Han TS**, Hur K, Xu G, Choi B, Okugawa Y, Toiyama Y, Oshima H, Oshima M, Lee HJ, Kim VN, Chang AN, Goel A, Yang HK. MicroRNA-29c mediates initiation of gastric carcinogenesis by directly targeting ITGB1. *Gut* 2015; **64**: 203-214 [PMID: 24870620 DOI: 10.1136/gutjnl-2013-306640]

106   **Su ZX**, Zhao J, Rong ZH, Wu YG, Geng WM, Qin CK. Diagnostic and prognostic value of circulating miR-18a in the plasma of patients with gastric cancer. *Tumour Biol* 2014; **35**: 12119-12125 [PMID: 25416437 DOI: 10.1007/s13277-014-2516-6]

107   **Xu L**, Hou Y, Tu G, Chen Y, Du YE, Zhang H, Wen S, Tang X, Yin J, Lang L, Sun K, Yang G, Tang X, Liu M. Nuclear Drosha enhances cell invasion via an EGFR-ERK1/2-MMP7 signaling pathway induced by dysregulated miRNA-622/197 and their targets LAMC2 and CD82 in gastric cancer. *Cell Death Dis* 2017; **8**: e2642 [PMID: 28252644 DOI: 10.1038/cddis.2017.5]

108   **Wu HH**, Lin WC, Tsai KW. Advances in molecular biomarkers for gastric cancer: miRNAs as emerging novel cancer markers. *Expert Rev Mol Med* 2014; **16**: e1 [PMID: 24456939 DOI: 10.1017/erm.2013.16]

109   **Liu HS**, Xiao HS. MicroRNAs as potential biomarkers for gastric cancer. *World J Gastroenterol* 2014; **20**: 12007-12017 [PMID: 25232237 DOI: 10.3748/wjg.v20.i34.12007]

110   **Korpal M**, Lee ES, Hu G, Kang Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. *J Biol Chem* 2008; **283**: 14910-14914 [PMID: 18411277 DOI: 10.1074/jbc.C800074200]

111   **Zhu X**, Lv M, Wang H, Guan W. Identification of circulating microRNAs as novel potential biomarkers for gastric cancer detection: a systematic review and meta-analysis. *Dig Dis Sci* 2014; **59**: 911-919 [PMID: 24337687 DOI: 10.1007/s10620-013-2970-9]

112   **Mitchell PS**, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* 2008; **105**: 10513-10518 [PMID: 18663219 DOI: 10.1073/pnas.0804549105]

113   **Sierzega M**, Kaczor M, Kolodziejczyk P, Kulig J, Sanak M, Richter P. Evaluation of serum microRNA biomarkers for gastric cancer based on blood and tissue pools profiling: the importance of miR-21 and miR-331. *Br J Cancer* 2017; **117**: 266-273 [PMID: 28641313 DOI: 10.1038/bjc.2017.190]

114   **Zhang Y**, Guan DH, Bi RX, Xie J, Yang CH, Jiang YH. Prognostic value of microRNAs in gastric cancer: a meta-analysis. *Oncotarget* 2017; **8**: 55489-55510 [PMID: 28903436 DOI: 10.18632/oncotarget.18590]

115   **Huang Z**, Zhu D, Wu L, He M, Zhou X, Zhang L, Zhang H, Wang W, Zhu J, Cheng W, Chen Y, Fan Y, Qi L, Yin Y, Zhu W, Shu Y, Liu P. Six Serum-Based miRNAs as Potential Diagnostic Biomarkers for Gastric Cancer. *Cancer Epidemiol Biomarkers Prev* 2017; **26**: 188-196 [PMID: 27756776 DOI: 10.1158/1055-9965.EPI-16-0607]

116   **Baniak N**, Senger JL, Ahmed S, Kanthan SC, Kanthan R. Gastric biomarkers: a global review. *World J Surg Oncol* 2016; **14**: 212 [PMID: 27514667 DOI: 10.1186/s12957-016-0969-3]

117   **Fang XY**, Pan HF, Leng RX, Ye DQ. Long noncoding RNAs: novel insights into gastric cancer. *Cancer Lett* 2015; **356**: 357-366 [PMID: 25444905 DOI: 10.1016/j.canlet.2014.11.005]

118   **Song H**, Sun W, Ye G, Ding X, Liu Z, Zhang S, Xia T, Xiao B, Xi Y, Guo J. Long non-coding RNA expression profile in human gastric cancer and its clinical significances. *J Transl Med* 2013; **11**: 225 [PMID: 24063685 DOI: 10.1186/1479-5876-11-225]

119   **Liu L**, Yan B, Yang Z, Zhang X, Gu Q, Yue X. ncRuPAR inhibits gastric cancer progression by down-regulating protease-activated receptor-1. *Tumour Biol* 2014; **35**: 7821-7829 [PMID: 24817013 DOI: 10.1007/s13277-014-2042-6]

120   **Chandra Gupta S**, Nandan Tripathi Y. Potential of long non-coding RNAs in cancer patients: From biomarkers to therapeutic targets. *Int J Cancer* 2017; **140**: 1955-1967 [PMID: 27925173 DOI: 10.1002/ijc.30546]

121   **Yuan CL**, Li H, Zhu L, Liu Z, Zhou J, Shu Y. Aberrant expression of long noncoding RNA PVT1 and its diagnostic and prognostic significance in patients with gastric cancer. *Neoplasma* 2016; **63**: 442-449 [PMID: 26925791 DOI: 10.4149/314_150825N45]

122   **Fan QH**, Yu R, Huang WX, Cui XX, Luo BH, Zhang LY. The has-miR-526b binding-site rs8506G>a polymorphism in the lincRNA-NR_024015 exon identified by GWASs predispose to non-cardia gastric cancer risk. *PLoS One* 2014; **9**: e90008 [PMID: 24595048 DOI: 10.1371/journal.pone.0090008]

123   **Koga A**, Aoyagi K, Imaizumi T, Miyagi M, Shirouzu K. Comparison between the gastric cancer cell line MKN-45 and the high-potential peritoneal dissemination gastric cancer cell line MKN-45P. *Kurume Med J* 2011; **58**: 73-79 [PMID: 22531121 DOI: 10.2739/kurumemedj.58.73]

124   **Pegtel DM**, Cosmopoulos K, Thorley-Lawson DA, van Eijndhoven MA, Hopmans ES, Lindenberg JL, de Gruijl TD, Würdinger T, Middeldorp JM. Functional delivery of viral miRNAs via exosomes. *Proc Natl Acad Sci USA* 2010; **107**: 6328-6333 [PMID: 20304794 DOI: 10.1073/pnas.0914843107]

125   **Skog J**, Würdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT Jr, Carter BS, Krichevsky AM, Breakefield XO. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol* 2008; **10**: 1470-1476 [PMID: 19011622 DOI: 10.1038/ncb1800]

126   **Valadi H**, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007; **9**: 654-659 [PMID: 17486113 DOI: 10.1038/ncb1596]

127   **Cheng L**, Sharples RA, Scicluna BJ, Hill AF. Exosomes provide a protective and enriched source of miRNA for biomarker profiling compared to intracellular and cell-free blood. *J Extracell Vesicles* 2014; **3**: [PMID: 24683445 DOI: 10.3402/jev.v3.23743]

128   **Khan S**, Bennit HF, Wall NR. The emerging role of exosomes in survivin secretion. *Histol Histopathol* 2015; **30**: 43-50 [PMID: 25020159 DOI: 10.14670/HH-30.43]

129   **Wang N**, Wang L, Yang Y, Gong L, Xiao B, Liu X. A serum exosomal microRNA panel as a potential biomarker test for gastric cancer. *Biochem Biophys Res Commun* 2017; **493**: 1322-1328 [PMID: 28986250 DOI: 10.1016/j.bbrc.2017.10.003]

130   **Tokuhisa M**, Ichikawa Y, Kosaka N, Ochiya T, Yashiro M, Hirakawa K, Kosaka T, Makino H, Akiyama H, Kunisaki C, Endo I. Exosomal miRNAs from Peritoneum Lavage Fluid as Potential Prognostic Biomarkers of Peritoneal Metastasis in Gastric Cancer. *PLoS One* 2015; **10**: e0130472 [PMID: 26208314 DOI: 10.1371/journal.pone.0130472]

131   **Watanabe Y**, Kim HS, Castoro RJ, Chung W, Estecio MR, Kondo K, Guo Y, Ahmed SS, Toyota M, Itoh F, Suk KT, Cho MY, Shen L, Jelinek J, Issa JP. Sensitive and specific detection of early gastric cancer with DNA methylation analysis of gastric washes. *Gastroenterology* 2009; **136**: 2149-2158 [PMID: 19375421 DOI: 10.1053/j.gastro.2009.02.085]

132   **Yamamoto H**, Watanabe Y, Oikawa R, Morita R, Yoshida Y, Maehata T, Yasuda H, Itoh F. BARHL2 Methylation Using Gastric Wash DNA or Gastric Juice Exosomal DNA is a Useful Marker For Early Detection of Gastric Cancer in an H. pylori-Independent Manner. *Clin Transl Gastroenterol* 2016; **7**: e184 [PMID: 27441821 DOI: 10.1038/ctg.2016.40]

133   **Virgilio E**, Giarnieri E, Giovagnoli MR, Montagnini M, Proietti A, D'Urso R, Mercantini P, Balducci G, Cavallini M. Gastric Juice MicroRNAs as Potential Biomarkers for Screening Gastric Cancer: A Systematic Review. *Anticancer Res* 2018; **38**: 613-616 [PMID: 29374683 DOI: 10.21873/anticanres.12265]

134   **Parsonnet J**, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, Sibley RK. Helicobacter pylori infection and the risk of gastric carcinoma. *N Engl J Med* 1991; **325**: 1127-1131 [PMID: 1891020 DOI: 10.1056/NEJM199110173251603]

135   **Hatakeyama M**. Structure and function of Helicobacter pylori CagA, the first-identified bacterial protein involved in human cancer. *Proc Jpn Acad Ser B Phys Biol Sci* 2017; **93**: 196-219 [PMID: 28413197 DOI: 10.2183/pjab.93.013]

136   **Yong X**, Tang B, Li BS, Xie R, Hu CJ, Luo G, Qin Y, Dong H, Yang SM. Helicobacter pylori virulence factor CagA promotes tumorigenesis of gastric cancer via multiple signaling pathways. *Cell Commun Signal* 2015; **13**: 30 [PMID: 26160167 DOI: 10.1186/s12964-015-0111-0]

137   **Saju P**, Murata-Kamiya N, Hayashi T, Senda Y, Nagase L, Noda S, Matsusaka K, Funata S, Kunita A, Urabe M, Seto Y, Fukayama M, Kaneda A, Hatakeyama M. Host SHP1 phosphatase antagonizes Helicobacter pylori CagA and can be downregulated by Epstein-Barr virus. *Nat Microbiol* 2016; **1**: 16026 [PMID: 27572445 DOI: 10.1038/nmicrobiol.2016.26]

138   **Pormohammad A**, Ghotaslou R, Leylabadlo HE, Nasiri MJ, Dabiri H, Hashemi A. Risk of gastric cancer in association with Helicobacter pylori different virulence factors: A systematic review and meta-analysis. *Microb Pathog* 2018; **118**: 214-219 [PMID: 29510208 DOI: 10.1016/j.micpath.2018.03.004]

139   **Altieri F**, Di Stadio CS, Federico A, Miselli G, De Palma M, Rippa E, Arcari P. Epigenetic alterations of gastrokine 1 gene expression in gastric cancer. *Oncotarget* 2017; **8**: 16899-16911 [PMID: 28129645 DOI: 10.18632/oncotarget.14817]

140   **Verma R**, Sharma PC. Next generation sequencing-based emerging trends in molecular biology of gastric cancer. *Am J Cancer Res* 2018; **8**: 207-225 [PMID: 29511593]

Bowel preparation quality scales for colonoscopy

David Kastenberg, Gerald Bertiger, Stuart Brogadir

David Kastenberg, Division of Gastroenterology and Hepatology, Department of Medicine, Thomas Jefferson University, Philadelphia, PA 19107, United States

Gerald Bertiger, Hillmont GI, Flourtown, PA 19031, United States

Stuart Brogadir, Medical Affairs, Ferring Pharmaceuticals, Inc, Parsippany, NJ 07054, United States

ORCID number: David Kastenberg (0000-0002-3995-2282); Gerald Bertiger (0000-0003-4731-2362); Stuart Brogadir (0000-0002-3608-7211).

Abstract

Colorectal cancer (CRC) is the third most common cancer and second leading cause of cancer-related death in the United States. Colonoscopy is widely preferred for CRC screening and is the most commonly used method in the United States. Adequate bowel preparation is essential for successful colonoscopy CRC screening. However, up to one-quarter of colonoscopies are associated with inadequate bowel preparation, which may result in reduced polyp and adenoma detection rates, unsuccessful screens, and an increased likelihood of repeat procedure. In addition, standardized criteria and assessment scales for bowel preparation quality are lacking. While several bowel preparation quality scales are referred to in the literature, these differ greatly in grading methodology and categorization criteria. Published reliability and validity data are available for five bowel preparation quality assessment scales, which vary in several key attributes. However, clinicians and researchers continue to use a variety of bowel preparation quality measures, including nonvalidated scales, leading to potential confusion and difficulty when comparing quality results among clinicians and across clinical trials. Optimal clinical criteria for bowel preparation quality remain controversial. The use of validated bowel preparation quality scales with stringent but simple scoring criteria would help clarify clinical trial data as well as the performance of colonoscopy in clinical practice related to quality measurements.

Key words: Colonoscopy; Bowel preparation; Aronchick scale; Ottawa Bowel Preparation Scale; Boston Bowel Preparation Scale

Core tip: Adequate bowel preparation is essential for proper visualization of the colonic mucosa to optimize lesion detection for a successful colonoscopy. Clinicians and researchers continue to use a variety of bowel preparation quality measures, including *de novo*, nonvalidated scales in clinical studies, leading to potential confusion, and creating difficulty when comparing bowel preparation quality results across clinical trials. Based on data evaluating different bowel preparation quality scales in the literature, and published criteria that define the most desirable measures to be used in such grading scales, the Boston Bowel Preparation Scale is currently recommended as standard.

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer, with an estimated risk of occurring in 1 of 18 persons during their lifetime, and is the second most common cause of cancer-related adult deaths in the United States[1,2]. Approximately 135000 new CRC cases and 50000 CRC deaths were projected to occur in 2017 in the United States[1]. For average risk individuals, the United States Preventive Services Task Force and other public health and professional medical bodies recommend CRC screening using colonoscopy, computerized tomography colonography, sigmoidoscopy, double-contrast barium enema, high-sensitivity guaiac or immunochemical fecal occult blood testing, or stool DNA testing (which is combined with immunochemical blood testing) beginning at the age of 50 years[2-4]. Colonoscopy is a preferred and the most widely used method for CRC screening in the United States[4-6], based on data showing this procedure is correlated with decreased CRC incidence and deaths, most likely through the detection and removal of premalignant polyps[7-10].

Adequate bowel preparation is essential to ensure sufficient visualization of the colonic mucosa and to optimize lesion detection for successful colonoscopy utilized for CRC screening[4,11]. However, study data indicate that up to one-quarter of colonoscopies may be conducted with inadequate bowel preparation[12,13], which is correlated with lower detection of polyps and adenomas *vs* adequate preparation (typically good/excellent quality)[12,14-16]. A meta-analysis of 27 studies found that inadequate bowel preparation for colonoscopy CRC screening reduced detection of small adenomas by 47% (OR = 0.53, CI: 0.46-0.62; $P < 0.001$) *vs* adequate

preparation (excellent/good/fair); this relationship was weaker but still significant for advanced adenomas (OR = 0.74, CI: 0.62-0.87; $P < 0.001$)[17]. Other studies have reported overall adenoma miss rates of 42%-48% for initial colonoscopies with inadequate or low-quality bowel preparation, based on findings at repeat colonoscopies[13,18]. Inadequate bowel preparation for colonoscopy may also result in prolonged procedures, more frequent repeat colonoscopies (at shorter than recommended intervals) and related increased costs, lower cecal intubation rates, and higher risk of electrocautery[6,11,19-21]. Studies in various international populations have found that inadequate cleansing is a factor in approximately 20%-70% of incomplete colonoscopies[22-25]. Professional gastroenterology societies recommend that clinical practices aim for minimum adequate bowel preparation rates of 85%-90%, and that bowel preparation quality be documented at the time of the screening[6,26].

Currently, no standard criteria or definition exists for qualitative terms such as "adequate", "inadequate", "excellent", "good", "fair", or "poor"; in some scales, adequate cleansing is defined as a composite of "good" and "excellent"[11,26]. Physician reporting on quality of bowel preparation, as well as overall colonoscopy quality, is highly inconsistent and often missing important elements, which may be attributable to lack of clear and consistent quality assessment standards[27]. Therefore, this review was conducted to summarize and discuss currently available bowel preparation quality scales and highlight the benefits of using a reliable and validated scale in both clinical practice and clinical trials of bowel preparation agents.

COMPONENTS OF A BOWEL PREPARATION QUALITY SCALE

Essential attributes of a dependable bowel preparation quality scale include reliability and validity[11]. Scale reliability involves the degree to which an instrument yields reproducible, or consistent, results for the same investigator (intrarater reliability) or among different investigators (interrater reliability), upon repeated testing[11,28]. Validity indicates how well the scale measures what it is designed to assess, which can be determined *via* several methods[29]. Validity may be assessed by comparison with results of other established and accepted scales used for the same purpose (*i.e.*, bowel preparation quality) in the same test population, referred to as construct validity. Scale validity may also be assessed by correlation with other specific criteria measuring relevant clinical outcomes, in this case, overall colonoscopy quality; this is referred to as criterion-related validity or predictive validity[29,30].

A commonly used criterion for overall quality of CRC screening colonoscopy is the adenoma detection rate (ADR), defined as the proportion of all CRC screening colonoscopies performed by a physician that reveal

at least one adenoma[6,31]. Studies have shown that colonoscopy ADR is strongly, inversely associated with reduced interval CRC rates (CRC diagnosed between the time of screening colonoscopy and the scheduled time of surveillance colonoscopy, which was up to 10 years)[32,33], and that increasing ADRs are correlated with reduced CRC incidence and mortality[34]. Some data also indicate that the polyp detection rate (PDR), the number of patients with at least one polyp removed during screening CRC, may also be a useful parameter of colonoscopy quality, particularly since it appears to correlate well with ADR[6]. However, use of the PDR raises additional questions related to the precise definition of "polyp". Other questions include whether the detection rates of sessile serrated polyps (SSPs), advanced adenomas, and multiple adenomas (as opposed to a "one and done" approach) should be used as key indicators of colonoscopy quality in addition to the ADR and PDR[6]. However, clinical data are insufficient for resolution of these issues, and no guidelines for correlation of bowel preparation quality with detection rates for SSPs, advanced adenomas, and multiple adenomas have yet been established[6]. Thus, ADR appears to be the best criterion currently available, as it is relatively easy to measure and has been shown to correlate with interval cancer rate.

The cecal intubation rate, an indicator of colonoscopy completion (reaching the cecum or anastomosis, if present), is another acknowledged quality measure[6,21,26]. Cecal intubation is essential for visualization of the proximal colon, including the caecum, where many colorectal neoplasms are located, in particular SSPs[6]. However, data on the independent association of cecal intubation rate with CRC risk have been mixed[32,35]. Longer withdrawal time is associated with higher ADR and higher SSP detection and is also considered a key criterion of colonoscopy quality secondary to ADR[6,36-38].

Another recommended criterion of colonoscopy quality is the level of adherence to recommended post-polypectomy and post-cancer surveillance intervals, which are based on study data[2,6,39,40]. The United States Multi-Society Task Force on Colorectal Cancer (USMSTFCC) has recommended that this criterion may serve as the overall indication of clinical adequacy of a bowel preparation[11]. Intra-procedure flushing and suctioning to remove fluid and semisolid debris is often performed during colonoscopy[11]. Therefore, the USMSTFCC recommends that bowel preparation quality should be assessed on withdrawal after washing and suctioning[11]. This criterion relates primarily to clinical adequacy, where washing and suctioning is taken into account, and is less relevant for the comparison of different bowel preparation agents, where pre-wash grading of bowel cleanse quality may better reflect preparation agent efficacy.

VALIDATED BOWEL PREPARATION SCALES

The most well established and commonly used validated bowel preparation quality scales in clinical trials include the Aronchick Scale[41,42], the Boston Bowel Preparation Scale (BBPS)[43-49], and the Ottawa Bowel Preparation Scale (OBPS)[50] (Table 1). Other instruments that have been validated, but are less commonly used, include the Harefield Cleansing Scale (HCS)[51] and the Chicago Bowel Preparation Scale (CBPS)[52] (Table 1). A summary of validation studies is found in Table 2.

Aronchick scale
The Aronchick Scale was the first bowel preparation quality scale to be evaluated for reliability[41,42]. This scale characterizes the percentage of the total colonic mucosal surface covered by fluid or stool, without scoring for separate colon segments, and is performed before washing or suctioning (Table 1). A validity study found that interobserver reliability kappa intraclass correlation coefficients (ICCs) were high for the cecum (0.76) and the total colon (0.77), but were reduced for the distal colon (0.31) and ascending colon segments[42]. The Aronchick Scale is one of the most commonly used validated bowel preparation quality scales in clinical trials and clinical practice.

Ottawa Bowel Preparation Scale
The OBPS measures mucosal cleanliness by colon segment, including the right colon, mid-colon, and recto-sigmoid colon, on a scale of 0 (excellent) to 4 (inadequate) for each (Table 1 and Figure 1), and is also scored before washing or suctioning[50]. However, in contrast to the Aronchick scale, the OBPS measures fluid quantity separately, with scores ranging from 0 (small volume) to 2 (large volume) for the total colon. Additionally, the OBPS does not tie scoring to subjective estimates of the percentage of the mucosa that is visible, which the investigators suggested might improve interobserver reliability (Table 1)[50]. In a study of reliability and validity compared with the Aronchick scale, the Pearson correlation coefficients for interobserver ratings were superior for the OBPS *vs* the Aronchick (0.89 *vs* 0.62, respectively; $P < 0.001$)[50]. Similarly, the kappa ICCs also significantly favored the OBPS *vs* the Aronchick scale [0.94 (95%CI: 0.91-0.96) *vs* 0.77 (95%CI: 0.65-0.84), respectively; $P < 0.001$]. Interrater consistency was found to be stronger with the OBPS *vs* the Aronchick scale, and reliability and agreement of the OBPS for the three different colon segments measured were very high, and not significantly different between segments (0.92 kappa, right colon; 0.88 kappa, mid-colon; 0.89 kappa, rectosigmoid; 0.94 kappa, total colon).

A prospective study of the OBPS aimed to identify an optimal cut-off score for bowel preparation adequacy/inadequacy in 211 patients undergoing colonoscopy at a single center[53]. The receiver operating characteristic (ROC) analysis used in this study found that an OBPS score cutoff of ≥ 8 identified inadequate bowel preparation with a sensitivity of 100% and a specificity of 91%. Another study in 150 consecutive patients undergoing colonoscopy reported strong concordance

Table 1 Validated bowel preparation scales

Scale name	Score	Rating/description	Other scale properties/characteristics
Aronchick Scale	1	Excellent: Small volume of liquid; > 95% of mucosa seen	Total score range: Minimum 1 (excellent) to maximum 5 (inadequate)
	2	Good: Clear liquid covering 5%-25% of mucosa, but > 90% of mucosa seen	Scoring performed before washing or suctioning
	3	Fair: Semisolid stool could not be suctioned or washed away, but > 90% of mucosa seen	No separate ratings for segments; global colon rating only
	4	Poor: Semisolid stool could not be suctioned or washed away and < 90% of mucosa seen	No threshold for adequate/inadequate provided
	5	Inadequate: Repeat preparation/screening needed	
Ottawa Bowel Preparation Scale (by colon segment)	0	Excellent: Mucosal detail clearly visible, almost no stool residue; if fluid present, it is clear, almost no stool residue	Total score (obtained by adding scores for each segment + total colon fluid score) range: Minimum 0 (excellent) to maximum 14 (inadequate)
	1	Good: Some turbid fluid or stool residue, but mucosal detail still visible without need for washing/suctioning	Scoring performed before washing or suctioning
	2	Fair: Some turbid fluid of stool residue obscuring mucosal detail; however, mucosal detail becomes visible with suctioning, washing not needed	Rates cleansing by colon segment: Right colon, mid-colon, and rectosigmoid colon (Figure 1)
	3	Poor: Stool present obscuring mucosal detail and contour; a reasonable view is obtained with suctioning and washing	No threshold for adequate/inadequate provided
	4	Inadequate: Solid stool obscuring mucosal detail and not cleared with washing and suctioning	
Ottawa Bowel Preparation Scale (total colon fluid)	0	Small amount of fluid	Total colon fluid score range: Minimum 0 (small amount of fluid) to maximum 2 (large amount of fluid)
	1	Moderate amount of fluid	Scoring performed before washing or suctioning
	2	Large amount of fluid	Single score for the total colon
			No threshold for adequate/inadequate provided
Boston Bowel Preparation Scale (by colon segment)	0	Unprepared colon segment with mucosa not seen because of solid stool that cannot be cleared	Total score (obtained by adding scores for each segment) range: Minimum 0 (very poor) to maximum 9 (excellent)
	1	Portion of mucosa of the colon segment seen, but other areas of segment not well seen because of staining, residual stool, and/or opaque liquid	Scoring performed after washing or suctioning
	2	Minor amount of residual staining, small fragments of stool, and/or opaque liquid, but mucosa of colon segment is well seen	Segments separately rated: Right colon (including cecum and ascending colon); transverse (includes hepatic and splenic flexures); and left colon (descending and sigmoid colon, and rectum)
	3	Entire mucosa of colon segment well seen, with no residual staining, small fragments of stool, or opaque liquid	Threshold optimally is total score of ≥ 6 AND ≥ 2 per segment
Harefield Cleansing Scale (by colon segment)	0	Irremovable, heavy, hard stools	Total score (obtained by adding scores for each segment) range: Minimum 0 (very bad) to maximum 20 (very good)
	1	Semisolid, only partially removable stools	Scoring performed after washing or suctioning
	2	Brown liquid/fully removable semi-solid stools	Segments separately rated: Rectum, sigmoid, left, transverse, right colon
	3	Clear liquid	Threshold for successful cleansing = Grade A: no segment scored < 3 or 4, or Grade B: ≥ 1 segment scored 2 but no segment < 2;
	4	Empty and clean	Unsuccessful cleansing = Grade C: ≥ 1 segment scored 1 but no segment < 1, or Grade D: ≥ 1 segment scored 0
Chicago Bowel Preparation Scale (by colon segment)	0	Unprepared colon segment with stool that cannot be cleared (> 15% of mucosa not seen)	Total score (obtained by adding scores for each segment) range: Minimum 0 (unprepared) to maximum 36 (excellent)
	5	Portion of mucosa in segment seen after cleaning, but up to 15% of the mucosa not seen because of retained material	Scoring performed before (fluid) and after (mucosal cleaning) washing or suctioning
	10	Minor residual material after cleaning, but mucosa of segment generally well seen	Segments separately rated: Right (cecum to mid-hepatic flexure), transverse (mid-hepatic flexure to mid-splenic flexure), and left colon (mid-splenic flexure to distal rectum)
	11	Entire mucosa of segment well seen after washing	No threshold for adequate/inadequate provided
	12	Entire mucosa of segment well seen before washing or suctioning	
Chicago Bowel Preparation Scale (total colon)	0	Little fluid (≤ 50 cc)	Total score range: Minimum 0 (little fluid) to maximum 3 (large amount of fluid)
	1	Minimal amount of fluid (51-150 cc)	Scoring performed before washing or suctioning
	2	Moderate amount of fluid (151-300 cc)	No threshold for adequate/inadequate provided
	3	Large amount of fluid (> 300 cc)	Not incorporated into total score for segments

Table 2 Reliability and validation data for bowel preparation scales

Scale	Study	Colons (*n*)	Raters (*n*)	Reliability	Validity
Aronchick	Aronchick[41], 2004	80	5	ICC values for: Total colon: 0.77 Cecum: 0.76 Distal colon: 0.31	NR
OBPS	Rostom *et al*[50], 2004	97	2	ICC values for: Right colon: 0.92 Mid colon: 0.88 Rectosigmoid colon: 0.89	Comparisons with Aronchick scale PCC: 0.89 OBPS *vs* 0.62 Aronchick ICC: 0.94 OBPS *vs* 0.77 Aronchick
	Chan *et al*[53], 2011	211	NR	NR	Cutoff scores for adequacy/inadequacy Optimal cutoff for inadequate $\geq$ 8: Sensitivity, 100%, specificity, 91%
	Martinato *et al*[54], 2013	150	NR	Ratings of physicians *vs* nurses: PCC: *r* = 0.60	Correlations with VAS PCC (physicians *vs* nurses): *r* = 0.60
	Lee *et al*[58], 2016	655	NA	NR	Comparison with BBPS for PDR and ADR PCC: *r* = -0.62 (*P* < 0.001); AUC of ROC analysis similar for PDR, ADR, right-sided adenomas, and SSAs
BBPS	Lai *et al*[47], 2009	633	22	ICC values: 0.74/0.77 wtd κ	PDR by score 40% for scores $\geq$ 5 *vs* 24% for scores < 5 (*P* < 0.02) Need for repeat CSP due to inadequate bowel prep 2% for scores $\geq$ 5 *vs* 73% for scores < 5 (*P* < 0.001) Correlation with colonoscope insertion time PCC: *r* = -0.16 (*P* < 0.003) Correlation with colonoscope withdrawal time PCC: *r* = -0.23 (*P* < 0.001)
	Calderwood *et al*[43], 2010	119	12	ICC values for: Total colon: 0.91 Right colon: 0.88 Transverse colon: 0.83 Left colon: 0.79	Correlations with ability to exclude polyps > 5 mm 100%, 88%, 82%, 33%, and 0% of physicians deemed bowel preparation adequate to exclude polyps > 5 mm at scores of $\geq$ 8, 7, 6, 5, and $\leq$ 4 respectively Correlations with surveillance recommendations after normal CSP Score < 5: 100% recommended $\leq$ 1 yr Scores 5-6: mean recommended interval 4.3 (± 3.9) yr Scores $\geq$ 7: 100% recommended 10 yr
	Calderwood *et al*[44], 2014	2516	74	NR	Physician-recommended CSP interval after negative CSP Scores $\geq$ 6 ($\geq$ 2 each segment): 90% recommended 10 yr Scores 0-2: 96% recommended $\leq$ 1 yr
	Schindler *et al*[49], 2016	3	40[1]	ICC values, all raters (all segment and total scores): 0.93	NR
	Gao *et al*[45], 2013	1012	13	ICC values: 0.987/0.671 wtd κ	PDR Scores $\geq$ 5 superior *vs* < 5 (35% *vs* 18%; *P* < 0.05)
	Kim *et al*[46], 2014	482	6	ICC values: Total colon: 0.90/0.63 wtd κ Right colon: 0.93/0.91 wtd κ Transverse colon: 0.88/0.86 wtd κ Left colon: 0.50/0.38 wtd κ	PDR Scores $\geq$ 8 superior *vs* scores < 8 (44.9% *vs* 33.0%; *P* = 0.04) Colonoscope withdrawal time PCC: *r* = -0.167 (*P* < 0.001) Colonoscope insertion time PCC: *r* = 0.018 (*P* = 0.695)
	Clark *et al*[57], 2016	438	4	ICC values by BBPS scores: 0 and 3: 1.0 2: 0.81 1: 0.80	ADR (> 5 mm) miss rates by BBPS score: 3: 5.6% 2: 5.2% 1: 15.9% Score of 2 noninferior to 3 for missed adenoma > 5 mm
HCS	Halphen *et al*[51], 2013	337	4	ICC value: 0.457 Test-retest κ values: Range, 0.33 to 0.85 Intrarater[2]: 0.28 to 0.64 Internal consistency[3]: 0.81, 0.86	Best score cutoff for satisfactory bowel preparation $\geq$ 2 for each segment: Sensitivity, 99% and specificity, 83% Correlation with Aronchick scale PCC: *r* = 0.833 AUC of ROC analysis (*vs* Aronchick scale scores) 0.945 for total colon
CBPS	Gerard *et al*[52], 2013	150	4[4]	ICC values for: Range, 0.624 to 0.702 for all segments	Correlations of scores with adequate cleansing Adequate: Scores of 25-36 ($\geq$ 95% of mucosa visualized) Inadequate: Scores of 0-24 (< 95% of mucosa visualized)

[1]Raters included endoscopy nurses (*n* = 17), gastroenterology faculty (*n* = 14), and gastroenterology fellows (*n* = 9); [2]Generalized κ for global agreement; [3]Cronbach's alpha; [4]Raters included three gastroenterologists and one physician's assistant. ADR: Adenoma detection rate; AUC: Area under the curve; BBPS: Boston Bowel Preparation Scale; CBPS: Chicago Bowel Preparation Scale; CSP: Colonoscopy; HCS: Harefield Cleansing Scale; ICC: Interobserver reliability kappa intraclass correlation coefficient; NA: Not applicable; NR: Not reported; OBPS: Ottawa Bowel Preparation Scale; PCC: Pearson correlation coefficient; PDR: Polyp detection rate; ROC: Receiver operating characteristic; SSA: Sessile serrated adenoma; VAS: Visual analogue scale; wtd: Weighted.

Figure 1 Bowel preparation quality scale segments. Depiction of bowel segments from validation study of Ottawa Bowel Preparation Scale[50]. Before washing or suctioning, each segment is scored on a scale of 0-4 for cleansing, and the total colon is scored for fluid quantity on a scale of 0-2. The total score ranges from 0 (excellent) to 14 (inadequate).

between the OBPS and a visual analogue scale measuring bowel cleansing among both nurses ($r = 0.8268$) and physicians ($r = 0.8095$, $P < 0.0001$ for both)[54]. The concordance in scoring between nurses and physicians was $r = 0.6010$; $P < 0.0001$.

Boston Bowel Preparation Scale
The BBPS has been validated in multiple clinical studies[11,47,55]. Developed in 2009, this scale was designed to address specific issues affecting bowel preparation quality and scoring: (1) The scale stipulates that scoring is to be conducted upon withdrawal and after all flushing and suctioning of fluid have been completed; (2) scoring is applied by colon segments, as in the OBPS, based on potential for variance in bowel preparation between segments; and (3) subjective, qualitative terms, such as excellent, good, fair, or poor, are replaced by numbered scores that are correlated to more clearly described colonic conditions, including features such as staining, liquid, and stool fragments (Table 1)[47]. Each segment of the colon is scored from 0 to 3, with higher scores indicating superior cleansing, and summed for a total score that can range from 0 to 9 (Table 1).

The initial validation study for the BBPS involved 633 CRC screening colonoscopies in a single center, and was applied by endoscopists who had undergone training on how to use the scale before participating in the study[47]. The median BBPS total score was 6. The ICC for interobserver agreement of total BBPS scores was 0.74 (95% predictive interval: 0.67-0.80), and the weighted kappa value for intraobserver agreement was 0.77 (95%CI: 0.66-0.87)[47]. Validity assessment was based on the correlations of BBPS scores with relevant clinical outcomes and more traditional scale categories, including "excellent", "good", "fair", "poor", or "unsatisfactory". Of the 633 patients who received a CRC screening colonoscopy, 243 (38%) had at least one polyp detected, and the PDR was significantly higher for patients with BBPS scores ≥ 5 *vs* those for patients with BBPS score < 5 (40% *vs* 24%, respectively; $P < 0.02$). The frequency of repeat colonoscopy attributable

to inadequate bowel preparation was significantly higher in patients with scores < 5 *vs* those with scores ≥ 5 (73% *vs* 2% of cases, respectively; $P < 0.001$). Total BBPS scores were inversely associated with colonoscopic insertion ($r = -0.16$; $P < 0.003$) and withdrawal times ($r = -0.23$; $P < 0.001$). In addition, a significant trend in mean BBPS score correlating with excellent, good, fair, poor, or unsatisfactory, as separately scored by the raters, was observed ($P < 0.001$ for trend).

A follow-up study investigated interobserver reliability and clinical outcome correlations of BBPS scores for individual segments, and relationship of scores to polyp detection in 119 screening colonoscopies rated by nine full-time faculty and three fellows at a single center[43]. All (100%) raters judged the bowel preparation adequate to exclude polyps > 5 mm with a ≥ 8 BBPS score, *vs* 88% of physicians when the score was 7, 82% when the score was 6, 33% when the score was 5, and 0% with a score of ≤ 4. Thus, a score of ≥ 6 was a particularly important threshold, since approximately 80% of physicians found the bowel preparation adequate at that score *vs* only one-third or less at BBPS scores of ≤ 5. In patients who had undergone a normal screening colonoscopy, a score of < 5 prompted all physicians to recommend repeat colonoscopy within one year, while a score of ≥ 7 was correlated with a recommendation for the next colonoscopy to occur in 10 years (among all physicians). BBPS segment scores were positively correlated with improved PDRs for the left and right colon, but no association was found for the transverse colon.

A further validation study was aimed at identifying a cut-off score for adequacy/inadequacy of bowel preparation[44]. This retrospective study of 2516 normal CRC screening colonoscopies performed by 74 endoscopists found that follow-up was recommended in 10 years for 90% of cases with a total BBPS score ≥ 6 in which all three segments had scores ≥ 2 ($n = 2295$), while 96% of examinations with total BBPS scores of 0-2 ($n = 26$) recommended follow-up within one year (Figure 2). Screenings with total scores of 3-5 ($n = 167$) had variable recommendations. Based on these findings, the investigators suggested that a total BBPS score of ≥ 6 and/or all segment scores ≥ 2 may serve as a standard definition of "adequate for 10-year follow-up"[44]. However, a prospective, observational study in a large, national endoscopic consortium found that inadequate single BBPS segment scores at the initial, average-risk screening colonoscopy were correlated with significantly greater risk of polyps at a second colonoscopy, suggesting that both a total score of ≥ 6 *and* all segment scores ≥ 2 should be required as an adequacy standard for 10-year follow-up[56]. This assessment was affirmed by a study in 438 colonoscopies in men, which found that BBPS segment scores of 2 or 3 (with 2 being noninferior to 3) was indicative of adequate bowel preparation for detection of adenomas > 5 mm, and for repeat colonoscopy at standard, guideline-recommended intervals (both parameters are USMSTFCC-recommended criteria for bowel preparation adequacy)[11,57].

Figure 2 Percentage of screening colonoscopy examinations in which 10-year follow-up was recommended after a negative colonoscopy, stratified by total Boston Bowel Preparation Scale Score[44].

Harefield Cleansing Scale

The HCS, developed in the 1990s, is scored by colon segment, as are the OBPS and BBPS[51]. Like the BBPS, the HCS is also scored after washing and suctioning are completed, and replaces qualitative terms (*e.g.*, "excellent" or "good") with direct descriptions of cleansing quality correlated with score numbers (Table 1)[51]. Grading is performed in five colon segments and ranges from 0-4 (higher numbers indicating better quality of cleanse) for each. Although total scores are derived by adding the separate segment scores, an "acceptable" score is possible only when the mucosa is 100% visible in all five colon segments. A validation study of the HCS compared with the Aronchick scale in 337 colonoscopies reviewed by four gastroenterologists found that there was a high degree of Pearson correlation between the two scales (r = 0.833), and the Spearman correlation coefficient was -0.778 (correlation is negative because improved cleanse quality is represented by different directions in the HCS and Aronchick scale)[51]. The ROC curve analysis *vs* the Aronchick scale showed an area under the curve of 0.945, and a sensitivity of 99% and specificity of 83% at the optimum score cut-off point. Interrater reliability analysis yielded an ICC of 0.457 (95%CI: 0.366-0.539). Cohen kappa scores for individual segments between investigators showed slight-to-fair agreement ranging from 0.15-0.27. Internal consistency was acceptable, based on a Cronbach alpha coefficient of 0.81, and the test-retest reliability assessment showed an overall kappa of 0.639. No analyses of correlations with relevant clinical outcomes such as the ADR or adherence to recall guidelines were performed, due to insufficient patient population.

Chicago Bowel Preparation Scale

Like the HCS, the CBPS was developed to address perceived limitations in other commonly used bowel preparation scales[52]. The main features of the scale are shown in Table 1. Scoring is performed both before and after washing or suctioning, and a separate fluid score is included as a secondary measure (not incorporated into the total score as in the OBPS). The total and fluid scoring categories were designed to measure both the quality of visualization and the intraprocedural effort required to clean the mucosa to attain adequate visualization. These parameters were intended to help clinicians assess the cleansing efficacy of different bowel preparations[52]. A CBPS validation study prospectively compared the results of the CBPS with the OBPS, the BBPS, and a theoretical, dichotomous scale that simply defined "adequate cleansing" as ability to see $\geq$ 95% of the mucosa (after it was cleansed), with "inadequacy" being defined as visibility in < 95% in 150 colonoscopies at a single center[52]. In this study, kappa coefficients for interrater agreement were higher for the CBPS (0.624-0.702) than the OBPS (0.493-0.655) and the BBPS (0.545-0.661), but these differences were not significant. Kappa coefficients for the total colon fluid scores for the CBPS and OBPS, and Pearson correlations coefficients for interrater agreement, were also similar. For the OBPS, scores from 8-10 were graded inadequate; for the BBPS, a score of $\leq$ 4 was graded inadequate; and for the CBPS, total scores $\leq$ 24 were graded inadequate. No clinically relevant parameters were assessed for validation in this study.

ADDITIONAL VALIDATED SCALE COMPARISON DATA

The OBPS and the BBPS were compared in a study that reviewed prospectively collected data from patients who underwent CRC screening or surveillance colonoscopies over a two-year period between August 2013 and July 2015[58]. Of the 655 colonoscopies, overall detection rates for polyp, adenoma, right-side adenoma, and sessile serrated adenoma (SSA) were 42.8%, 32.8%, 20.8%, and 1.2%, respectively. A significant Pearson correlation was observed between the two scales (P < 0.001).

However, the ROC curves for the OBPS *vs* the BBPS were not significantly different for the detection rates, respectively, for polyps (0.550 *vs* 0.513), adenoma (0.544 *vs* 0.519), right-side adenoma (0.469 *vs* 0.516), and SSA (0.712 *vs* 0.790). The investigators concluded that the choice of either the OBPS or the BBPS may not strongly affect the measurement of bowel preparation quality.

DISCUSSION

Quality scales
All currently available bowel preparation quality scales are imperfect, have limitations, and are dependent upon subjective descriptions of luminal contents expressed as categories ("excellent", "good", *etc*.) or numbers, depending on the scale utilized. A standard, fully validated, and universally accepted scale for use in clinical practice and trials has not yet been established. Among the scales, the Aronchick scale is the most well-known and widely used clinically and in clinical trials to date; however, this scale rates cleanse quality of the colon as a whole and provides no details regarding differences between individual segments.

Colon segments cleansing
Guidance is somewhat vague for clinicians regarding grading of the entire colon when individual segments are suboptimally cleansed. This issue may arise more often in the proximal colon, which is harder to clean than other segments and more likely to contain flat lesions such as sessile serrated polyps/adenomas[50,51]. Segment-specific bowel preparation quality scales, such as the OBPS or BBPS, may provide a clearer distinction between cleanse quality of the proximal colon compared with other segments. Furthermore, establishing a minimum acceptable score for adequacy within each colon segment, as has been done for the BBPS, is helpful in determining overall colon cleansing adequacy. A BBPS validation study provided information used to create an "adequate cleansing" threshold score of at least 2 in each of three colon segments.

Need for washing and suctioning
Grading before or after washing and suctioning is another important factor which differs between scales. Many clinicians are using the Aronchick scale incorrectly, as they grade the bowel preparation as good or fair after washing and suctioning. While scales that grade cleanse quality after washing may correlate better with quality measures such as ADR, or the likelihood of an alteration in CRC screening follow-up recommendations, scales that grade before washing can provide a better reflection of a bowel preparation product's efficacy independent of the endoscopist. Similarly, the OBPS gives points based on the total fluid in the colon, which leads to inaccurate grading if using water immersion/exchange.

The OBPS entails scoring by colon segments, thus accounting for variation by segment in bowel prep-

aration quality/visibility; however, it also incorporates the presence of luminal fluid before suctioning[11,50]. The OBPS validation data are largely dependent on correlations with the Aronchick scale, which itself has limited validation and may not correlate with ADR[50]. The BBPS differs in several key aspects from the Aronchick and OBPS scales[47]. To begin, it requires washing and suctioning to be completed before the bowel preparation is graded[47]. The HCS requires rating only after completion of flushing and suctioning, providing a score for the entire colon as well as for individual segments[51,52].

Grading scales validity and reliability
The reliability and validation data for BBPS is more extensive compared with the Aronchick and OBPS scales and include good supporting data correlating scores with key clinical outcomes. These validation studies have provided information to create a threshold for adequate cleansing of a score of at least 2 in each of three colon segments[44,57]. It should also be noted, however, that one study found no significant difference between the BBPS and OBPS regarding key indicators of colonoscopy quality, such as the PDR and ADR, in screening or surveillance colonoscopy[58]. Concerning the HCS and CBPS, each has reported acceptable reliability data, although the CBPS validation study was based on findings from only two raters[51,52]. While the HCS validation assessment was the only one to provide test-retest and internal consistency data for reliability, its validity evaluation was based only on correlations with the Aronchick scale[51]. Although the CBPS was compared with the OBPS and BBPS, no correlations of this scale with key clinical outcomes, such as ADR and adherence to screening and surveillance colonoscopy intervals, have been reported[52]. The CBPS has more specific definitions and requires measurement of fluid suctioned (Table 1), but the complexity may be challenging for the clinician to assess correctly; thus, it may not easily translate to clinical practice. Hence, the usefulness of these scales for clinical practice or trials remains unclear.

Several unique, nonvalidated bowel preparation scales have been developed for use in trials of agents including oral sulfate solution (OSS) (Suprep®, Braintree Laboratories, Braintree, MA, United States)[59], OSS plus sulfate-free electrolyte lavage solution (Suclear®, Braintree Laboratories, Braintree, MA, United States)[60], and polyethylene glycol electrolyte solution plus ascorbic acid (MoviPrep®, Salix Pharmaceuticals, Bridgewater, NJ, United States)[59,61,62]. The grading criteria used in these study- and product-specific scales often differ greatly from validated scales.

The substantial ramification of using nonvalidated scales is illustrated by a *post hoc* analysis of data from two sodium picosulfate and magnesium citrate (P/MC) clinical trials. Investigators analyzed the data from the studies after altering the definition of "adequate" in the Aronchick scale, which had been used in the original trials, to more closely resemble what has been used

in some studies utilizing nonvalidated scales[55]. With this revised definition, > 98% of all P/MC patients were considered responders, compared with 79%-87% using the original OBPS and Aronchick scale categorization criteria. Multiple studies have used more than one validated scale from among the Aronchick, OBPS, and BBPS scales for assessment of bowel preparation quality, providing additional comparative data[63-70]. Generally, the results of these trials have been concordant in assessment of bowel preparation quality, with similar mean total scores being reported for overall quality, and similar comparative assessments of different bowel preparations.

While scales for assessment of bowel preparation quality for CRC screening colonoscopy have improved, establishing a standard, validated scale is essential to optimize CRC colonoscopy screening. The Boston bowel preparation scale has several limitations, but appears nonetheless to be the best available option, and is therefore recommended as the current standard for use in clinical practice. Given the importance preparation plays in multiple colonoscopy quality measures, including the need to repeat the procedure when cleansing is inadequate, it may be advantageous for clinicians to adopt one language to describe cleansing quality. The continued use of multiple scales with varying criteria may undermine the validity of study findings and the accuracy of colonoscopy for CRC screening and surveillance.

For colonoscopy clinical trials, the use of different, and sometimes nonvalidated, scales across studies is one of many reasons comparisons between studies is fraught with difficulties. By incorporating a standard, validated grading scale, we may ensure that the findings are generalizable and comparable with other studies and facilitate progress in the development of future bowel preparations. Future developments in bowel preparation quality assessment are likely to involve establishment of an improved "gold standard" and further refinement of the accuracy of quality assessment. Continued improvement of quality standards for CRC prevention, further studies of ADR and withdrawal time, and recommended years of follow-up are also warranted.

REFERENCES

1   **Siegel RL**, Miller KD, Fedewa SA, Ahnen DJ, Meester RGS, Barzi A, Jemal A. Colorectal cancer statistics, 2017. *CA Cancer J Clin* 2017; **67**: 177-193 [PMID: 28248415 DOI: 10.3322/caac.21395]

2   **US Preventive Services Task Force**, Bibbins-Domingo K, Grossman DC, Curry SJ, Davidson KW, Epling JW Jr, García FAR, Gillman MW, Harper DM, Kemper AR, Krist AH, Kurth AE, Landefeld CS, Mangione CM, Owens DK, Phillips WR, Phipps MG, Pignone MP, Siu AL. Screening for colorectal cancer: US Preventive Services Task Force recommendation statement. *JAMA* 2016; **315**: 2564-2575 [PMID: 27304597 DOI: 10.1001/jama.2016.5989]

3   **Winawer SJ**, Zauber AG, Fletcher RH, Stillman JS, O'brien MJ, Levin B, Smith RA, Lieberman DA, Burt RW, Levin TR, Bond JH, Brooks D, Byers T, Hyman N, Kirk L, Thorson A, Simmang C, Johnson D, Rex DK. Guidelines for colonoscopy surveillance after polypectomy: a consensus update by the US Multi-Society Task Force on Colorectal Cancer and the American Cancer Society. *CA Cancer J Clin* 2006; **56**: 143-159; quiz 184-185 [PMID: 16737947 DOI: 10.3322/canjclin.56.3.143]

4   **Lieberman D**, Ladabaum U, Cruz-Correa M, Ginsburg C, Inadomi JM, Kim LS, Giardiello FM, Wender RC. Screening for colorectal cancer and evolving issues for physicians and patients: a review. *JAMA* 2016; **316**: 2135-2145 [PMID: 27893135 DOI: 10.1001/jama.2016.17418]

5   **U.S. Preventive Services Task Force**. Screening for colorectal cancer: U.S. Preventive Services Task Force recommendation statement. *Ann Intern Med* 2008; **149**: 627-637 [PMID: 18838716 DOI: 10.7326/0003-4819-149-9-200811040-00243]

6   **Rex DK**, Schoenfeld PS, Cohen J, Pike IM, Adler DG, Fennerty MB, Lieb JG 2nd, Park WG, Rizk MK, Sawhney MS, Shaheen NJ, Wani S, Weinberg DS. Quality indicators for colonoscopy. *Gastrointest Endosc* 2015; **81**: 31-53 [PMID: 25480100 DOI: 10.1016/j.gie.2014.07.058]

7   **Austin H**, Henley SJ, King J, Richardson LC, Eheman C. Changes in colorectal cancer incidence rates in young and older adults in the United States: what does it tell us about screening. *Cancer Causes Control* 2014; **25**: 191-201 [PMID: 24249437 DOI: 10.1007/s10552-013-0321-y]

8   **Chen C,** Stock C, Hoffmeister M, Brenner H. Public health impact of colonoscopy use on colorectal cancer mortality in Germany and the United States. *Gastrointest Endosc* 2018; **87**: 213-221.e2 [PMID: 28431951 DOI: 10.1016/j.gie.2017.04.005]

9   **Murphy CC**, Sandler RS, Sanoff HK, Yang YC, Lund JL, Baron JA. Decrease in incidence of colorectal cancer among individuals 50 years or older after recommendations for population-based screening. *Clin Gastroenterol Hepatol* 2017; **15**: 903-909.e6 [PMID: 27609707 DOI: 10.1016/j.cgh.2016.08.037]

10   **Zauber AG**, Winawer SJ, O'Brien MJ, Lansdorp-Vogelaar I, van Ballegooijen M, Hankey BF, Shi W, Bond JH, Schapiro M, Panish JF, Stewart ET, Waye JD. Colonoscopic polypectomy and long-term prevention of colorectal-cancer deaths. *N Engl J Med* 2012; **366**: 687-696 [PMID: 22356322 DOI: 10.1056/NEJMoa1100370]

11   **Johnson DA**, Barkun AN, Cohen LB, Dominitz JA, Kaltenbach T, Martel M, Robertson DJ, Boland CR, Giardello FM, Lieberman DA, Levin TR, Rex DK; US Multi-Society Task Force on Colorectal Cancer. Optimizing adequacy of bowel cleansing for colonoscopy: recommendations from the US multi-society task force on colorectal cancer. *Gastroenterology* 2014; **147**: 903-924 [PMID: 25239068 DOI: 10.1053/j.gastro.2014.07.002]

12   **Harewood GC**, Sharma VK, de Garmo P. Impact of colonoscopy preparation quality on detection of suspected colonic neoplasia. *Gastrointest Endosc* 2003; **58**: 76-79 [PMID: 12838225 DOI: 10.1067/mge.2003.294]

13   **Lebwohl B**, Kastrinos F, Glick M, Rosenbaum AJ, Wang T, Neugut AI. The impact of suboptimal bowel preparation on adenoma miss rates and the factors associated with early repeat colonoscopy. *Gastrointest Endosc* 2011; **73**: 1207-1214 [PMID: 21481857 DOI: 10.1016/j.gie.2011.01.051]

14   **Froehlich F**, Wietlisbach V, Gonvers JJ, Burnand B, Vader JP. Impact of colonic cleansing on quality and diagnostic yield of colonoscopy: the European Panel of Appropriateness of Gastrointestinal Endoscopy European multicenter study. *Gastrointest Endosc* 2005; **61**: 378-384 [PMID: 15758907 DOI: 10.1016/S0016-5107(04)02776-2]

15   **Sherer EA**, Imler TD, Imperiale TF. The effect of colonoscopy preparation quality on adenoma detection rates. *Gastrointest Endosc* 2012; **75**: 545-553 [PMID: 22138085 DOI: 10.1016/j.gie.2011.09.022]

16   **Adler A**, Wegscheider K, Lieberman D, Aminalai A, Aschenbeck J, Drossel R, Mayr M, Mroß M, Scheel M, Schröder A, Gerber K, Stange G, Roll S, Gauger U, Wiedenmann B, Altenhofen L, Rosch T. Factors determining the quality of screening colonoscopy: a prospective study on adenoma detection rates, from 12,134 examinations (Berlin colonoscopy project 3, BECOP-3). *Gut* 2013; **62**: 236-241 [PMID: 22442161 DOI: 10.1136/gutjnl-2011-300167]

17   **Sulz MC**, Kröger A, Prakash M, Manser CN, Heinrich H, Misselwitz B. Meta-analysis of the effect of bowel preparation on adenoma detection: early adenomas affected stronger than advanced adenomas. *PLoS One* 2016; **11**: e0154149 [PMID: 27257916 DOI: 10.1371/journal.pone.0154149]

18   **Chokshi RV**, Hovis CE, Hollander T, Early DS, Wang JS. Prevalence of missed adenomas in patients with inadequate bowel preparation

on screening colonoscopy. *Gastrointest Endosc* 2012; **75**: 1197-1203 [PMID: 22381531 DOI: 10.1016/j.gie.2012.01.005]

19   **Rex DK**, Imperiale TF, Latinovich DR, Bratcher LL. Impact of bowel preparation on efficiency and cost of colonoscopy. *Am J Gastroenterol* 2002; **97**: 1696-1700 [PMID: 12135020 DOI: 10.1111/j.1572-0241.2002.05827.x]

20   **Smith CL,** Roy A, Kalra AP, Daskalakis C, Kastenberg D. Adenoma detection on repeat colonoscopy after previous inadequate preparation. *J Gastroenterol Hepatol Res* 2013; **2**: 911-917

21   **ASGE Standards of Practice Committee**, Saltzman JR, Cash BD, Pasha SF, Early DS, Muthusamy VR, Khashab MA, Chathadi KV, Fanelli RD, Chandrasekhara V, Lightdale JR, Fonkalsrud L, Shergill AK, Hwang JH, Decker GA, Jue TL, Sharaf R, Fisher DA, Evans JA, Foley K, Shaukat A, Eloubeidi MA, Faulx AL, Wang A, Acosta RD. Bowel preparation before colonoscopy. *Gastrointest Endosc* 2015; **81**: 781-794 [PMID: 25595062 DOI: 10.1016/j.gie.2014.09.048]

22   **Rees CJ**, Thomas Gibson S, Rutter MD, Baragwanath P, Pullan R, Feeney M, Haslam N; British Society of Gastroenterology, the Joint Advisory Group on GI Endoscopy, the Association of Coloproctology of Great Britain and Ireland. UK key performance indicators and quality assurance standards for colonoscopy. *Gut* 2016; **65**: 1923-1929 [PMID: 27531829 DOI: 10.1136/gutjnl-2016-312044]

23   **Koido S**, Ohkusa T, Nakae K, Yokoyama T, Shibuya T, Sakamoto N, Uchiyama K, Arakawa H, Osada T, Nagahara A, Watanabe S, Tajiri H. Factors associated with incomplete colonoscopy at a Japanese academic hospital. *World J Gastroenterol* 2014; **20**: 6961-6967 [PMID: 24944489 DOI: 10.3748/wjg.v20.i22.6961]

24   **Parente F**, Marino B, Crosta C. Bowel preparation before colonoscopy in the era of mass screening for colo-rectal cancer: a practical approach. *Dig Liver Dis* 2009; **41**: 87-95 [PMID: 18676211 DOI: 10.1016/j.dld.2008.06.005]

25   **Audibert C,** Perlaky A, Glass D. Global perspective on colonoscopy use for colorectal cancer screening: A multi-country survey of practicing colonoscopists. *Contemp Clin Trials Commun* 2017; **7**: 116-121 [PMID: 29696175 DOI: 10.1016/j.conctc.2017.06.008]

26   **Rembacken B**, Hassan C, Riemann JF, Chilton A, Rutter M, Dumonceau JM, Omar M, Ponchon T. Quality in screening colonoscopy: position statement of the European Society of Gastrointestinal Endoscopy (ESGE). *Endoscopy* 2012; **44**: 957-968 [PMID: 22987217 DOI: 10.1055/s-0032-1325686]

27   **Sharma RS**, Rossos PG. A Review on the Quality of Colonoscopy Reporting. *Can J Gastroenterol Hepatol* 2016; **2016**: 9423142 [PMID: 27446877 DOI: 10.1155/2016/9423142]

28   **Carmines EG,** Zeller RA. Reliability and validity assessment. London: SAGE Publications, 1979 [DOI: 10.4135/9781412985642]

29   **Karras DJ**. Statistical methodology: II. Reliability and validity assessment in study design, Part B. *Acad Emerg Med* 1997; **4**: 144-147 [PMID: 9043544 DOI: 10.1111/j.1553-2712.1997.tb03723.x]

30   **Nunnally JC,** Bernstein IH. Psychometric theory. 3rd ed. New York: McGraw-Hill, 1994

31   **Clark BT**, Rustagi T, Laine L. What level of bowel prep quality requires early repeat colonoscopy: systematic review and meta-analysis of the impact of preparation quality on adenoma detection rate. *Am J Gastroenterol* 2014; **109**: 1714-1723; quiz 1724 [PMID: 25135006 DOI: 10.1038/ajg.2014.232]

32   **Kaminski MF**, Regula J, Kraszewska E, Polkowski M, Wojciechowska U, Didkowska J, Zwierko M, Rupinski M, Nowacki MP, Butruk E. Quality indicators for colonoscopy and the risk of interval cancer. *N Engl J Med* 2010; **362**: 1795-1803 [PMID: 20463339 DOI: 10.1056/NEJMoa0907667]

33   **Corley DA**, Jensen CD, Marks AR, Zhao WK, Lee JK, Doubeni CA, Zauber AG, de Boer J, Fireman BH, Schottinger JE, Quinn VP, Ghai NR, Levin TR, Quesenberry CP. Adenoma detection rate and risk of colorectal cancer and death. *N Engl J Med* 2014; **370**: 1298-1306 [PMID: 24693890 DOI: 10.1056/NEJMoa1309086]

34   **Kaminski MF**, Wieszczy P, Rupinski M, Wojciechowska U, Didkowska J, Kraszewska E, Kobiela J, Franczyk R, Rupinska M, Kocot B, Chaber-Ciopinska A, Pachlewski J, Polkowski M, Regula J. Increased rate of adenoma detection associates with reduced risk of colorectal cancer and death. *Gastroenterology* 2017; **153**: 98-105

[PMID: 28428142 DOI: 10.1053/j.gastro.2017.04.006]

35   **Baxter NN**, Sutradhar R, Forbes SS, Paszat LF, Saskin R, Rabeneck L. Analysis of administrative data finds endoscopist quality measures associated with postcolonoscopy colorectal cancer. *Gastroenterology* 2011; **140**: 65-72 [PMID: 20854818 DOI: 10.1053/j.gastro.2010.09.006]

36   **Barclay RL**, Vicari JJ, Doughty AS, Johanson JF, Greenlaw RL. Colonoscopic withdrawal times and adenoma detection during screening colonoscopy. *N Engl J Med* 2006; **355**: 2533-2541 [PMID: 17167136 DOI: 10.1056/NEJMoa055498]

37   **Simmons DT**, Harewood GC, Baron TH, Petersen BT, Wang KK, Boyd-Enders F, Ott BJ. Impact of endoscopist withdrawal speed on polyp yield: implications for optimal colonoscopy withdrawal time. *Aliment Pharmacol Ther* 2006; **24**: 965-971 [PMID: 16948808 DOI: 10.1111/j.1365-2036.2006.03080.x]

38   **Lee RH**, Tang RS, Muthusamy VR, Ho SB, Shah NK, Wetzel L, Bain AS, Mackintosh EE, Paek AM, Crissien AM, Saraf LJ, Kalmaz DM, Savides TJ. Quality of colonoscopy withdrawal technique and variability in adenoma detection rates (with videos). *Gastrointest Endosc* 2011; **74**: 128-134 [PMID: 21531410 DOI: 10.1016/j.gie.2011.03.003]

39   **Imperiale TF**, Glowinski EA, Lin-Cooper C, Larkin GN, Rogge JD, Ransohoff DF. Five-year risk of colorectal neoplasia after negative screening colonoscopy. *N Engl J Med* 2008; **359**: 1218-1224 [PMID: 18799558 DOI: 10.1056/NEJMoa0803597]

40   **Brenner H**, Chang-Claude J, Seiler CM, Hoffmeister M. Long-term risk of colorectal cancer after negative colonoscopy. *J Clin Oncol* 2011; **29**: 3761-3767 [PMID: 21876077 DOI: 10.1200/JCO.2011.35.9307]

41   **Aronchick CA**. Bowel preparation scale. *Gastrointest Endosc* 2004; **60**: 1037-1038; author reply 1038-1039 [PMID: 15605036 DOI: 10.1016/S0016-5107(04)02213-8]

42   **Aronchick CA,** Lipshutz WH, Wright SH, DuFrayne F, Bergman G. Validation of an instrument to assess colon cleansing. *Am J Gastroenterol* 1999; **94**: 2667

43   **Calderwood AH**, Jacobson BC. Comprehensive validation of the Boston Bowel Preparation Scale. *Gastrointest Endosc* 2010; **72**: 686-692 [PMID: 20883845 DOI: 10.1016/j.gie.2010.06.068]

44   **Calderwood AH**, Schroy PC 3rd, Lieberman DA, Logan JR, Zurfluh M, Jacobson BC. Boston Bowel Preparation Scale scores provide a standardized definition of adequate for describing bowel cleanliness. *Gastrointest Endosc* 2014; **80**: 269-276 [PMID: 24629422 DOI: 10.1016/j.gie.2014.01.031]

45   **Gao Y**, Lin JS, Zhang HD, Lin MX, Cheng CS, Wu SZ. Pilot validation of the Boston Bowel Preparation Scale in China. *Dig Endosc* 2013; **25**: 167-173 [PMID: 23368700 DOI: 10.1111/j.1443-1661.2012.01356.x]

46   **Kim EJ**, Park YI, Kim YS, Park WW, Kwon SO, Park KS, Kwak CH, Kim JN, Moon JS. A Korean experience of the use of Boston bowel preparation scale: a valid and reliable instrument for colonoscopy-oriented research. *Saudi J Gastroenterol* 2014; **20**: 219-224 [PMID: 25038207 DOI: 10.4103/1319-3767.136950]

47   **Lai EJ**, Calderwood AH, Doros G, Fix OK, Jacobson BC. The Boston bowel preparation scale: a valid and reliable instrument for colonoscopy-oriented research. *Gastrointest Endosc* 2009; **69**: 620-625 [PMID: 19136102 DOI: 10.1016/j.gie.2008.05.057]

48   **Mittal S**. The Boston bowel preparation scale: reliable not only for colonoscopy-oriented research but clinical practice also. *Gastrointest Endosc* 2010; **71**: 221 [PMID: 20105483 DOI: 10.1016/j.gie.2009.04.031]

49   **Schindler AE**, Chan WW, Laborde CJ, Obstein KL. Reliability of the Boston Bowel Preparation Scale in the endoscopy nurse population. *Clin Gastroenterol Hepatol* 2016; **14**: 775-776 [PMID: 25460559 DOI: 10.1016/j.cgh.2014.11.011]

50   **Rostom A**, Jolicoeur E. Validation of a new scale for the assessment of bowel preparation quality. *Gastrointest Endosc* 2004; **59**: 482-486 [PMID: 15044882 DOI: 10.1016/S0016-5107(03)02875-X]

51   **Halphen M**, Heresbach D, Gruss HJ, Belsey J. Validation of the Harefield Cleansing Scale: a tool for the evaluation of bowel cleansing quality in both research and clinical practice. *Gastrointest*

Endosc 2013; **78**: 121-131 [PMID: 23531426 DOI: 10.1016/j.gie.2013.02.009]

52   **Gerard DP**, Foster DB, Raiser MW, Holden JL, Karrison TG. Validation of a new bowel preparation scale for measuring colon cleansing for colonoscopy: the chicago bowel preparation scale. *Clin Transl Gastroenterol* 2013; **4**: e43 [PMID: 24304940 DOI: 10.1038/ctg.2013.16]

53   **Chan M,** Birnstein E, Patel N, Chan L, Laine L, Kline M. Ottawa score of 8 or greater is an optimal cut-off score for inadequate bowel preparation. *Am J Gastroenterol* 2011; **106**: S431-S432

54   **Martinato M,** Krankovic I, Caccaro R, Scacchi M, Cesaro R, Marzari F, Colombara F, Compagno D, Judet S, Sturniolo GC, D'Incà R. Assessment of bowel preparation for colonoscopy: comparison between different tools and different healthcare professionals. *Dig Liver Dis* 2013; **45S**: S195-S196 [DOI: 10.1016/S1590-8658(13)60558-7]

55   **Parmar R**, Martel M, Rostom A, Barkun AN. Validated scales for colon cleansing: a systematic review. *Am J Gastroenterol* 2016; **111**: 197-204; quiz 205 [PMID: 26782820 DOI: 10.1038/ajg.2015.417]

56   **Kluge MA**, Williams JL, Wu CK, Jacobson BC, Schroy PC 3rd, Lieberman DA, Calderwood AH. Inadequate Boston Bowel Preparation Scale scores predict the risk of missed neoplasia on the next colonoscopy. *Gastrointest Endosc* 2018; **87**: 744-751 [PMID: 28648575 DOI: 10.1016/j.gie.2017.06.012]

57   **Clark BT**, Protiva P, Nagar A, Imaeda A, Ciarleglio MM, Deng Y, Laine L. Quantification of adequate bowel preparation for screening or surveillance colonoscopy in men. *Gastroenterology* 2016; **150**: 396-405; quiz e14-e15 [PMID: 26439436 DOI: 10.1053/j.gastro.2015.09.041]

58   **Lee YJ,** Kim ES, Cho KB, Park KS, Lee Jy, Lee YS, Choi WY, Kwon TH. SU1731 Comparison of Ottawa and Boston bowel preparation scales for adenoma detection rate. *Gastrointest Endosc* 2016; **83** Suppl: AB413 [DOI: 10.1016/j.gie.2016.03.1043]

59   **Di Palma JA**, Rodriguez R, McGowan J, Cleveland Mv. A randomized clinical study evaluating the safety and efficacy of a new, reduced-volume, oral sulfate colon-cleansing preparation for colonoscopy. *Am J Gastroenterol* 2009; **104**: 2275-2284 [PMID: 19584830 DOI: 10.1038/ajg.2009.389]

60   **Rex DK**, DiPalma JA, McGowan J, Cleveland Mv. A comparison of oral sulfate solution with sodium picosulfate: magnesium citrate in split doses as bowel preparation for colonoscopy. *Gastrointest Endosc* 2014; **80**: 1113-1123 [PMID: 25028274 DOI: 10.1016/j.gie.2014.05.329]

61   **Bitoun A**, Ponchon T, Barthet M, Coffin B, Dugué C, Halphen M; Norcol Group. Results of a prospective randomised multicentre controlled trial comparing a new 2-L ascorbic acid plus polyethylene glycol and electrolyte solution vs. sodium phosphate solution in patients undergoing elective colonoscopy. *Aliment Pharmacol Ther* 2006; **24**: 1631-1642 [PMID: 17094774 DOI: 10.1111/j.1365-2036.2006.03167.x]

62   **Ell C**, Fischbach W, Bronisch HJ, Dertinger S, Layer P, Rünzi M, Schneider T, Kachel G, Grüger J, Köllinger M, Nagell W, Goerg KJ, Wanitschke R, Gruss HJ. Randomized trial of low-volume PEG solution versus standard PEG + electrolytes for bowel cleansing before colonoscopy. *Am J Gastroenterol* 2008; **103**: 883-893 [PMID: 18190651 DOI: 10.1111/j.1572-0241.2007.01708.x]

63   **Bertiger G,** Epstein M, Walker AH, Almansa C, Dahdal D. Comparison of bowel preparation quality scales used in randomized controlled trials of patients undergoing colonoscopy. Poster presented at: the American College of Gastroenterology 2016 Annual Scientific Meeting and Postgraduate Course; 2016 Oct 14-19; Las Vegas, NV. Poster 1000

64   **Katz PO**, Rex DK, Epstein M, Grandhi NK, Vanner S, Hookey LC, Alderfer V, Joseph RE. A dual-action, low-volume bowel cleanser administered the day before colonoscopy: results from the SEE CLEAR II study. *Am J Gastroenterol* 2013; **108**: 401-409 [PMID: 23318484 DOI: 10.1038/ajg.2012.441]

65   **Rex DK**, Katz PO, Bertiger G, Vanner S, Hookey LC, Alderfer V, Joseph RE. Split-dose administration of a dual-action, low-volume bowel cleanser for colonoscopy: the SEE CLEAR I study. *Gastrointest Endosc* 2013; **78**: 132-141 [PMID: 23566639 DOI: 10.1016/j.gie.2013.02.024]

66   **Samarasena JB**, Muthusamy VR, Jamal MM. Split-dosed MiraLAX/Gatorade is an effective, safe, and tolerable option for bowel preparation in low-risk patients: a randomized controlled study. *Am J Gastroenterol* 2012; **107**: 1036-1042 [PMID: 22565162 DOI: 10.1038/ajg.2012.115]

67   **Brahmania M**, Ou G, Bressler B, Ko HK, Lam E, Telford J, Enns R. 2 L versus 4 L of PEG3350 + electrolytes for outpatient colonic preparation: a randomized, controlled trial. *Gastrointest Endosc* 2014; **79**: 408-416.e4 [PMID: 24206747 DOI: 10.1016/j.gie.2013.08.035]

68   **Gweon TG**, Kim SW, Noh YS, Hwang S, Kim NY, Lee Y, Lee SW, Lee SW, Lee JY, Lim CH, Hun Kim H, Kim JS, Kyung Cho Y, Myung Park J, Seok Lee I, Myung-Gyu Choi. Prospective, randomized comparison of same-day dose of 2 different bowel cleanser for afternoon colonoscopy: picosulfate, magnesium oxide, and citric acid versus polyethylene glycol. *Medicine* (Baltimore) 2015; **94**: e628 [PMID: 25837751 DOI: 10.1097/MD.0000000000000628]

69   **Sharara AI**, Chalhoub JM, Beydoun M, Shayto RH, Chehab H, Harb AH, Mourad FH, Sarkis FS. A customized mobile application in colonoscopy preparation: a randomized controlled trial. *Clin Transl Gastroenterol* 2017; **8**: e211 [PMID: 28055031 DOI: 10.1038/ctg.2016.65]

70   **Belsey J**, Crosta C, Epstein O, Fischbach W, Layer P, Parente F, Halphen M. Meta-analysis: the relative efficacy of oral bowel preparations for colonoscopy 1985-2010. *Aliment Pharmacol Ther* 2012; **35**: 222-237 [PMID: 22112043 DOI: 10.1111/j.1365-2036.2011.04927.x]

Current practices and future prospects for the management of gallbladder polyps: A topical review

R Stephen McCain, Anna Diamond, Claire Jones, Helen G Coleman

R Stephen McCain, Centre for Public Health, Institute of Clinical Sciences, Queens University Belfast, Belfast BT12 6BJ, United Kingdom

Anna Diamond, Ulster Hospital, South Eastern Health and Social Care Trust, Belfast BT16 1RH, United Kingdom

Claire Jones, Mater Hospital, Belfast Health and Social Care Trust, Queens University Belfast, Belfast BT12 6BJ, United Kingdom

Helen G Coleman, Centre for Public Health, Queen's University Belfast, Belfast BT12 6BJ, United Kingdom

ORCID number: R Stephen McCain (0000-0001-5357-1622); Anna Diamond (0000-0003-0195-2639); Claire Jones (0000-0003-2961-7744); Helen G Coleman (0000-0003-4872-7877).

Abstract

A gallbladder polyp is an elevation of the gallbladder mucosa that protrudes into the gallbladder lumen. Gallbladder polyps have an estimated prevalence in adults of between 0.3%-12.3%. However, only 5% of polyps are considered to be "true" gallbladder polyps, meaning that they are malignant or have malignant potential. The main radiological modality used for diagnosing and surveilling gallbladder polyps is transabdominal ultrasonography. However, evidence shows that other modalities such as endoscopic ultrasound may improve diagnostic accuracy. These are discussed in turn during the course of this review. Current guidelines recommend cholecystectomy for gallbladder polyps sized 10 mm and greater, although this threshold is lowered when other risk factors are identified. The evidence behind this practice is relatively low quality. This review identifies current gaps in the available evidence and highlights the necessity for further research to enable better decision making regarding which patients should undergo cholecystectomy, and/or radiological follow-up.

Key words: Gallbladder polyps; Gallbladder cancer; True polyps; Pseudo polyps

Core tip: Evidence for the optimum management of gallbladder polyps is lacking. The main imaging modality used for diagnosis and follow-up is transabdominal ultrasound, but some studies suggest improved accuracy with

endoscopic ultrasound. Other imaging modalities lack evidence. Surgical management involves cholecystectomy and the general consensus is that polyps 10 mm and greater should undergo surgery. However, this is an arbitrary cut-off and high-quality evidence to support this is lacking. Lowering the threshold for cholecystectomy when patients have additional risk factors for gallbladder malignancy may improve the cancer detection rate in polyps smaller than 10 mm, but again, the evidence behind this is lacking.

INTRODUCTION

A gallbladder polyp is an elevation of the gallbladder mucosa that protrudes into the gallbladder lumen[1,2]. Gallbladder polyps have an estimated prevalence of approximately 5% in the global population, but only 5% of these are considered to be "true" gallbladder polyps[3,4]. The majority of gallbladder polyps are detected incidentally on radiological imaging or histological examination after cholecystectomy. However, a small number of patients with gallbladder polyps may be symptomatic and present with acute cholecystitis due to the polyp obstructing the cystic duct, or cholangitis due to fragments of the polyp breaking off and travelling down into in the bile duct[2,5]. The majority of gallbladder polyps are classified as "pseudo"-polyps, as displayed in Figure 1. "Pseudo"-polyps have no malignant potential and do not require any follow-up or intervention, whereas "true" gallbladder polyps, which include adenocarcinomas or adenomas require surgical removal[2]. Although adenomas are benign, they have malignant potential and there is some evidence to suggest they may follow the adenoma-carcinoma sequence as seen in colorectal cancer[6,7].

Gallbladder cancer is the 20th most common cancer in the world and there are an estimated 178100 new cases diagnosed each year[8]. The highest incidences of gallbladder cancer are seen in South America and Asia, whilst lower incidences are seen in developed regions such as North America and the United Kingdom[9]. For example, the incidence of gallbladder cancer in Chile and Bolivia is 12.8 and 10.9 per 100000 population respectively, whereas in the United Kingdom and North America the incidence is 1.6 and 1.5 per 100000 people[9,10]. The staging of gallbladder cancer as per the American Joint Committee on Cancer 8th edition, ranges from stage 0 to stage 4b. Stage 0 describes carcinoma in-situ when the cancer involves the mucosa only as seen in early polyp cancers, while stage 4b indicates

lymph node involvement of 4 or more lymph nodes (N2 disease) or the presence of metastatic disease[11]. Survival in gallbladder cancer patients varies significantly from an 80% 5-year survival in those with in-situ disease, declining to only 8% when lymph nodes are involved, and 2% for patients with stage 4b disease[11]. These figures demonstrate the importance of identifying malignant and pre-malignant polyps to enable early treatment to prevent cancer spread or development of malignancy.

It should be noted that once detected, surgical removal of all gallbladder polyps is not appropriate, given that the majority of polyps are "pseudo"-polyps with no malignant potential and there is a significant risk associated with surgery. In patients with "true" gallbladder polyps, laparoscopic cholecystectomy is the surgical option preferred, although in patients with larger polyps, open cholecystectomy is recommended[12,13]. The risks associated with surgery include damage to intra-abdominal structures during port insertion, bile duct injury (between 0.3% and 1%) and bile leak[14,15]. Furthermore, surgical intervention to repair a bile duct injury and endoscopic retrograde cholangio-pancreatography (ERCP) to manage a bile leak are associated with significant mortality, cholangitis, biliary cirrhosis, pancreatitis, perforation and haemorrhage[16,17].

This review discusses the current evidence that exists regarding the management of gallbladder polyps. Given the low incidence of true polyps within all gallbladder polyps identified, coupled with the high mortality associated with gallbladder cancer and the risk of complications associated with cholecystectomy, it is essential to differentiate between "pseudo"- polyps and true polyps to enable appropriate management. The use of imaging modalities assists with the decision-making process and this review discusses the benefits and shortcomings of the imaging modalities used for identifying and following up gallbladder polyps.

THE ROLE OF DIFFERENT IMAGING MODALITIES IN GALLBLADDER POLYP DIAGNOSIS

Radiological imaging plays the main role in the diagnosis and decision making for the management of gallbladder polyps. The ideal imaging modalities should have three key features. Firstly, they should be able to accurately diagnose polyps and differentiate them from gallstones, sludge, or folds of the gallbladder mucosa. Secondly, "true" polyps need to be differentiated from "pseudo"-polyps, as the latter are benign with no malignant potential and therefore do not require any intervention or follow-up. Thirdly, the size of polyps need to be measured accurately as this is currently the most important factor which determines if patients should undergo cholecystectomy, radiological follow up or cease to be followed up. Given that some patients with gallbladder polyps will require follow-up for many years, it is also important that

Figure 1 Spider diagram showing the classification of gallbladder polyps.

the imaging modality is acceptable to patients and incurs minimal radiation exposure.

Accurate imaging will prevent unnecessary surgery and ensure true polyps which do not fall into the size criteria for surgical removal category are identified during follow-up. The benefits and shortcomings of different imaging modalities are discussed below. The main modalities discussed include ultrasonography, computed tomography and magnetic resonance imaging.

Transabdominal ultrasonography

Trans abdominal ultrasound (TAUS), encompasses conventional ultrasound (CUS), high-resolution ultrasound (HRUS), three-dimensional ultrasound and contrast enhanced ultrasound (CEUS). CUS and HRUS are easily accessible, cheap, non-invasive tests[18] and are the most widely used modalities for diagnosing and following up gallbladder polyps. However, other studies have been performed to assess the effectiveness of the other forms of ultrasonography mentioned above[18,19].

Ultrasonography is operator dependent and results can be limited by increased body mass index, in particular truncal obesity[20]. Polyp echogenicity is examined to distinguish between "true" polyps and "pseudo"- polyps and the presence of a fixed lesion helps to distinguish between polyps and gallstones. However, in some cases gallstones may be impacted in the gallbladder wall and be incorrectly labelled as a polyp[2]. Features that suggest the presence of a "pseudo"- polyp include a "comet tail" which arises posterior to the lesion but this is not identifiable in all "pseudo"- polyps[21].

CUS uses a low-frequency transducer between 2 and 5MHz but despite this has demonstrated good specificity (71%-98%) and sensitivity (50%-90%) for diagnosing all types of gallbladder polyps[22]. In the same systematic review, CUS had a sensitivity of 47%-67% and specificity of 36-100% for diagnosing malignancy[22] and in polyps 10mm or greater in size, the sensitivity and specificity for identifying malignancy was 78%-100% and 52%-87%, respectively[22].

However, shortcomings in CUS have been reported, for example in a single study by French *et al*[23] which compared histopathology reports from cholecystectomy specimens with findings from the CUS report found that imaging only identified 50% of polyps. This group concluded that CUS should not be used for following up gallbladder polyps[23].

HRUS operates at a higher frequency than CUS (5-7 MHz) but a lower frequency than endoscopic ultrasound (EUS) (5-12 MHz) and therefore theoretically has a better diagnostic accuracy than CUS but is less accurate than EUS[24]. It does however have the benefit over EUS, in that it is a non-invasive procedure. Kim *et al*[24] demonstrated that HRUS is more accurate than CUS at staging the T-stage of gallbladder cancer and was more accurate for identifying hypoechoic foci in neoplastic polyps which has previously been shown to be a strong predictive factor for neoplastic gallbladder polyps[24,25]. More studies however are required which compare the sensitivity and specificity of CUS and HRUS.

One study has compared HRUS, endoscopic ultrasound (EUS), and computed tomography (CT) in diagnosing and staging gallbladder polyps in 144 patients who all had a polyp greater than 10 mm in size[26]. Diagnostic sensitivities for malignancy were highest in HRUS, compared to the other two modalities and specificity was the same when using EUS and HRUS[26]. The drawback from this study however is that the applicability of this technique to smaller gallbladder polyps remains unknown and polyps of less than 10 mm are diagnostically most difficult group to assess. Furthermore, HRUS was not compared to CUS, which is currently the most commonly used imaging modality.

3D-US is an emerging modality which eliminates the operator dependency seen in 2-dimensional CUS. Research for this imaging modality is minimal but a study of 80 patients with gallbladder polyps found that there was agreement in the diagnosis in 89% of cases when both techniques were applied[27]. This study however found that 3D-US did have difficulty detecting polyps less than 4mm, but it is predicted that as technology continues to evolve this issue will decline in future[27]. Current research therefore does not support the routine use of 3D-US for evaluating gallbladder polyps.

Several small studies have looked at the use of contrast media to improve the diagnostic accuracy of CUS. Contrast aids radiologists to differentiate normal from abnormal conditions. Numata *et al*[28] used galactose palmitic acid contrast injection to assess 35 polyps which were larger than 10 mm in size. Using the criteria of tumour enhancement and tortuous type tumour vessels, this technique had 91% accuracy at identifying malignancy. The downside to this study however, is that it did not compare contrast-enhanced ultrasonography with CUS[28]. Zheng *et al*[29] did compare the two modalities in a study of 116 patients with gallbladder polyps, and

found that CEUS was useful for improving diagnostic accuracy in polyps greater than 10 mm, but not less than 10 mm in size.

Endoscopic ultrasound

EUS works at a higher frequency as described above and enables the transducer to be in closer proximity to the target tissue therefore, hypothetically improving diagnostic accuracy[24]. It is however, an invasive examination associated with a small risk of bleeding and upper gastrointestinal perforation and presents a higher risk of complications than all forms of TAUS[30].

A systematic review has found EUS to have a greater sensitivity (67%-86%) and specificity (84%-91%) for diagnosing malignancy in polyps than CUS[22]. A single study by Sugiyama *et al*[31] compared EUS and CUS in 58 patients who had undergone cholecystectomy. All polyps were 20 mm or less in size, and EUS was more accurate at differentiating between true and "pseudo"- polyps than CUS (97% *vs* 76%). Cheon *et al*[32] however, found that although EUS was more successful at identifying true polyps in those with diameters of 11 mm and greater (83% *vs* 64%), there was not the same success in polyps of diameter 10 mm and less (80% *vs* 72%). Therefore, this imaging technique may play a role in decreasing the number of unnecessary cholecystectomies in larger gallbladder polyps, but more research needs to be done investigating its role in smaller polyps, for which the management is most controversial.

Two studies have been performed looking at the role of contrast- enhanced EUS (CE-EUS) in diagnosing gallbladder polyps. Park studied 34 patients who had a cholecystectomy for gallbladder polyps and found that CE-EUS when attempting to distinguish adenomatous polyps from cholesterol polyps had a sensitivity of 75% and specificity of 66.6%. Unfortunately, in this study CE-EUS was not compared to any other imaging modality. Choi *et al*[33] however compared EUS with CE-EUS and found that diagnostic accuracy was slightly improved with the latter.

Other methods including the use of real time colour Doppler flow EUS has been used to try and improve the diagnostic accuracy of EUS. Kim *et al*[34] found that the presence of a strong colour Doppler flow in a study 115 patients who underwent cholecystectomy for gallbladder polyps may help predict the presence of neoplastic polyps and therefore further research is warranted.

Computed tomography

CT imaging is widely used in the staging of gallbladder adenocarcinoma[2]. However, some research has been performed to assess if it may also play a role in differentiating between true and "pseudo"- polyps and for long-term surveillance[35]. The accuracy of CT imaging was assessed in 31 patients with polypoid lesions of the gallbladder of 3cm or less. The CT diagnosis was accurate in 87% of cases however, only 5 polyps were less than 11 mm and therefore this study provides us with limited evidence regarding the role of CT in this

group of patients[35]. Lou *et al*[36] assessed the accuracy of CT biliary cystoscopy in 32 patients and found that CUS accurately detected polyps in 96.9% of cases compared to 93.8% for CT.

This evidence would suggest that CT imaging is best used in staging larger, suspicious malignant polyps, rather than for diagnostic purposes and follow-up, due to lack of superiority to CUS demonstrated in studies to date.

Magnetic resonance imaging

Minimal research has been performed looking at the role of MRI in differentiating between benign and malignant gallbladder polyps. In a small study, Irie *at al*[37] demonstrated in 10 benign polyps and 13 malignant polyps that the ADC values of the malignant lesions were significantly lower than that seen in the benign lesions. They concluded that diffusion-weighted MR imaging may play a role in diagnosing benign and malignant polyps[37]. However, further research is warranted to establish if MRI can improve the accuracy of diagnosing gallbladder polyps.

Other imaging modalities

Other imaging modalities have been considered in small single studies. One study has shown that positive emission tomography can differentiate between benign and malignant disease but more research is needed[2]. Results from a study examining the role of percutaneous transhepatic cholecystoscopy were promising but this is an invasive procedure with significant risk and is difficult for patients to tolerate[2]. Finally, intravenous cholecystography has shown to be of no benefit to date, compared with current imaging modalities[18].

After studying the evidence, TAUS and in particular CUS and HRUS would appear to be the most appropriate imaging modality for detecting gallbladder polyps. Although some studies looking at the role of other forms of ultrasonography in managing gallbladder polyps appear promising, there is still not enough evidence to introduce these modalities into routine practice for the management of gallbladder polyps. Evidence for smaller gallbladder polyps is of particularly low quality. In cases of clear uncertainty however, additional imaging modalities may be deployed to help the clinician in their decision-making process. The role of CT is evident in staging gallbladder cancer but due to a lack of high-quality studies examining a role in gallbladder polyps and the high radiation exposure associated with this imaging, it is not appropriate for either the diagnosis or follow-up of gallbladder polyps.

FACTORS INFLUENCING THE MANAGEMENT OF GALLBLADDER POLYPS

Polyp size

Studies have shown that malignant polyps in general

tend to be larger than benign polyps[5,22]. Kwon *et al*[5] reported in their study of 291 patients that malignant polyps had a mean size of 27.97+/-2.46 mm compared to 8.56+/-0.36 mm in the benign group. Currently, the polyp size on radiological imaging is the biggest contributing factor to the management plan for gallbladder polyps. Multiple retrospective studies have found the risk of malignancy rises sharply from 10 mm and upwards, and the general consensus is that patients with polyps of 10 mm or greater should be treated with cholecystectomy[19,22,38]. Although this is the accepted practice, evidence for this recommendation lacks quality. The most up-to-date guidelines published by the European Society of Gastrointestinal and Abdominal Radiology (ESGAR) support this approach but two recent systematic reviews demonstrate that although the majority of malignant polyps are over 10 mm in diameter, there are a significant number of both malignant polyps or polyps with malignant potential under this sizing threshold[19,22,38].

Babu *et al*[22] performed a systematic review which included 43 studies, of which 20 provided information on the size and histology of 2347 polyps. Of these, 356 were classified as true polyps, of which 228 were malignant - and 29 of these were between 5-10 mm but none below the 5 mm size. Bhatt *et al*[38] in their systematic review also demonstrated that there were a significant number of malignant polyps under 10 mm in size but the probability of malignancy when a polyp was 4.15 mm or smaller was approximately zero. These two large studies demonstrate that although the majority of true polyps are over 10 mm there are a significant number of true polyps under this cut off which will be missed if cholecystectomy is only performed for polyps greater than 10 mm.

Several authors have suggested a change in this cut off with some suggesting polyps of 6 mm and larger should undergo cholecystectomy whilst others have felt that the cut off should be increased to 12mm[39,40]. The argument for lowering the threshold carries more weight, as demonstrated by the findings in the systematic reviews discussed above. The counter-argument of lowering the threshold is that by offering cholecystectomy to those patients with polyps below 10 mm, a greater number of patients may be put through an unnecessary operation associated with significant risk of complications. It has therefore been proposed that polyps under 10 mm should undergo surveillance, based on their size unless significant risk factors are present in which case cholecystectomy should be offered[19].

Surveillance

Polyp surveillance aims to provide a safety net for those patients with true polyps that cannot be differentiated from "pseudo"- polyps on radiological investigations and are under 10 mm in diameter. It is hypothesised that "true" polyps will undergo faster growth, and by careful follow-up these can be identified early and removed[22]. Guidelines state that polyps which reach 10 mm in size

or increase in size by 2 mm at follow up transabdominal ultrasonography are recommended to be removed surgically[19]. However, evidence to support this practice is lacking.

There is no consensus on the size of polyps that require follow up, or the frequency or duration of follow up. The most recent set of guidelines published by ESGAR states that patients with polyps of 6-9 mm should be followed up more extensively than patients with polyps of less than 6 mm[19]. Several studies support 6 mm as a lower limit cut-off for less extensive follow up, but go a step further by suggesting the cessation of follow up in polyps less than 6 mm[41,42]. However, this has been contradicted by multiple studies which have found true polyps to be less than 6 mm in size and a single case report that has shown that a 5 mm polyp transformed into a 20 mm carcinoma over a period of two years[22,38,43]. The evidence would suggest that all polyps between 4-10 mm should be followed up equally as although the risk reduces with size, there is still a significant number of true polyps between 4 mm and 6mm. Although no malignant polyps have been shown to be below 4 mm there is still a risk of adenomas and these polyps therefore would still require follow up but on a less frequent basis[22].

The recommended follow up for patients with gallbladder polyps depends on the size of the polyps and the presence of risk factors for malignancy, but opinions differ and the evidence base informing these guidelines is relatively limited. For example, Babu *et al*[22] recommend that the follow up of polyps 5-10 mm should be two scans at six month intervals and following this the surveillance plan should be tailored for individual patients. The ESGAR group recommend that in polyps of 6-9 mm, after two initial six monthly scans there should be yearly scans up to 5 years. However, in polyps under 6 mm there should be imaging at 1, 3 and 5 years but if he patient has risk factors for malignancy there should be more extensive follow-up as those seen for polyps of 6-9 mm with no risk factors[22].

Follow up imaging may have a limited benefit as only a small number of polyps actually change in size during follow up. Babu *et al*[22] identified 10 studies which looked at the follow up of gallbladder polyps between six months and seven years. They found that only 7.6% of polyps increased in size and Bhatt *et al*[38] also found that that 93% of polyps did not change in size during follow up. Neither study stated if growth was more likely to be seen in in pseudo or true polyps and this was supported in a third systematic review[44]. Although there is a lack of evidence comparing growth patterns between pseudo-polyps and true polyps, small individual studies have shown that both can undergo sudden growth[2].

RISK FACTORS FOR GALLBLADDER POLYP MALIGNANCY

As discussed above the main determining factor for

Table 1 Summary of evidence for association between potential risk factors and malignant gallbladder polyps

Risk factor	Direction of association	Strength of association	Related notable findings	Key references
Age	Positive	Probability of malignancy was 20.7% in those patients older than 50	This systematic review studied polyps less than 10 mm only	[38]
Sessile morphology	Positive	Probability of malignancy was 13.9% in sessile compared to pedunculated polyps	This systematic review studied polyps less than 10 mm only	[38]
Presence of gallstones	Inconclusive	Aldouri *et al*[47] found increased risk of malignancy with gallstones (HR = 3.2, 95%CI: 1.42-7.22) but Park *et al*[39] found no difference (*P* = 0.27)	There is no strong evidence to suggest there is a definite association	[39,47]
Indian Ethnicity	Positive	HR = 12.92 (95%CI: 3.77-44.29) This shows a significant HR but the width of the CI's are noted.	This is the only study to compare risk between Indian ethnicity and Caucasian race	[47]
Primary sclerosing cholangitis	Positive	40%-60% of polyps in patients with PSC were malignant	33% of those with benign polyps had associated dysplasia	[56]

gallbladder malignancy is the presence of a polyp greater than 10 mm in size. However, not all polyps under 10 mm are benign and therefore it is important to identify risk factors to enable the clinician to have a higher suspicion for malignancy and therefore perform cholecystectomy below the 10 mm threshold. These potential risk factors are discussed below and summarised in Table 1.

Number of polyps
Evidence is mixed on whether solitary polyps are more likely to be malignant compared to the presence of multiple polyps. In a systematic review by Bhatt *et al*[38], the probability of malignancy in a polyp under 10 mm if it was solitary was 4.3% higher compared to when multiple polyps were present. The authors did not deem this to incur a high enough risk to suggest cholecystectomy in all patients with a solitary polyp under 10mm. Perhaps this is the most useful study as the authors look at the risk exclusively in the 5-9 mm group and it is this cohort in which the evidence is weakest[38]. A study by Kwon *et al*[5] also found that malignant polyps were more likely to be solitary (*P* = 0.02), but this study only patients who had gallbladder polyps greater than 10 mm. Several other studies however have demonstrated no association between a solitary polyp and malignancy. For example, Park *et al*[39] in a study of 689 patients found that 60% of benign polyps were solitary and 76% of malignant polyps were benign and this was not significantly different (*P* = 0.11).

Although the probability of malignancy is not high enough to recommend cholecystectomy in all solitary polyps, the presence of a solitary polyp should be considered in combination with other risk factors for malignancy as discussed below.

Sessile morphology
Single studies such as that performed by Kwon *et al*[5] have demonstrated that patients with gallbladder polyps of sessile morphology have a higher risk of malignancy compared to those with pedunculated polyps (OR: 7.70; 95%CI: 2.48-23.95). In the systematic review by Bhatt

et al[38], malignant polyps under 10 mm were also more likely to be sessile in nature and the probability of malignancy was 13.9% in these patients but cholecystectomy was not recommended. However, if there was a solitary sessile polyp, the probability of malignancy was 24.8% and cholecystectomy was recommended[38]. Although Bhatt *et al*[38] do not recommend cholecystectomy based on sessile morphology alone, the most recent guidelines by the ESGAR group use the strength of this evidence to recommend cholecystectomy for all sessile polyps under between 6 mm and 9 mm.

Age
The risk of most cancers increases with age and a similar pattern is seen for gallbladder cancer. Multiple case series support this but the cut off for an increased risk of malignancy varies significantly between 50 and 65 years old[13,38,39,45]. For example, Park *et al*[39] identified age 57 years and older as a risk factor for malignancy, but in this study one patient who was only 37 years old had a malignant polyp of 10 mm and the one patient who had a malignant polyp under 10 mm in size was only 50 years old. Furthermore, Sarkut *et al*[46] found that there was an increased likelihood of malignancy in patients aged 50 and over, but again this was not exclusive as one patient under 50 had a malignant polyp. The only study to date that looks at the contribution of age to risk of malignancy in polyps solely under 10 mm was performed by Bhatt *et al*[38]. They found that when the polyp was less than 10 mm and the patient was over 50 that the probability of malignancy was 20.7%, and therefore cholecystectomy was recommended[38]. The ESGE group used this evidence to conclude that if patients are aged 50 and have polyps of 6-9 mm they should undergo cholecystectomy[19].

Presence of gallstones
The evidence considering the impact of concurrent gallstones and the risk of malignancy in gallbladder polyps varies significantly and is of relatively low quality. Aldouri *et al*[47] found that if gallstones were present there was an increased risk of malignancy (HR: 3.2;

95%CI: 1.42-7.22) but Park *et al*[39] found that there was no association between the presence of gallstones and malignancy (*P* = 0.27). In those patients with symptoms due to gallstones, cholecystectomy is already recommended and therefore the decision-making process is simple. However, the evidence is not strong enough to suggest cholecystectomy should be performed in all cases with dual pathology.

Ethnicity

As discussed earlier, gallbladder cancer incidence varies significantly between countries. A study by Aldouri *et al*[47] carried out in the United Kingdom demonstrated that in 5391 patients who underwent cholecystectomy, the risk of malignancy was almost 13 times higher in the Indian population compared to the Caucasian population (HR: 12.92; 95%CI: 3.77-44.29). This is the only study to date which compares risk between different ethnic groups, however the ESGAR felt the evidence was so compelling that their guidelines state that in patients of Indian ethnicity and a polyp between 6-9 mm they should undergo cholecystectomy[19]. Further research needs to be performed comparing other ethnic groups to determine if there should be a lower threshold for cholecystectomy in different ethnicities.

Primary sclerosing cholangitis

Primary sclerosing cholangitis (PSC) is a recognised risk factor for a gallbladder polyp malignancy, and cholecystectomy is currently recommended in these patients who have a gallbladder polyp irrespective of the polyp size[48]. The largest study to date including 286 PSC patients, found that in 18 patients with a gallbladder polyp, 10 had a malignancy in polyps as small as 5 mm whilst in 9 patients who had no mass lesion they still had dysplasia of the gallbladder[49]. Furthermore, in a case series of 4 patients with PSC and gallbladder polyps, all were shown to have malignant disease including in two polyps under 10 mm in size[50]. Other evidence is less compelling, including a study by Eaton *et al*[51] who found that in 14 patients with PSC and polyps only two were malignant. This group concluded that polyps under 8 mm were less likely to be malignant and in this group and follow up should be applied. Given the presence of research such as this further research would be justified. The difficulty will be recruiting enough patients with both pathologies.

Tumour markers

Limited research has been performed to assess if there is a role for tumour markers in the pre-operative evaluation of gallbladder polyps. The two markers focused on to date has been CEA and CA19-9 but no correlation between malignancy and elevated markers has been found. In a case series of 291 patients, Kwon *et al*[5] found no difference in pre-operative CEA or CA19-9 levels in the benign or malignant groups. Indeed, the CEA level was elevated in more benign cases (5.7%)

than malignant cases (2.9%). When comparing the CA19-9 levels, there were 4.9% of benign group who had a raised level and 8.6% of malignant group had a raised level[5]. There is no sufficient evidence to show that tumour markers will assist in the decision-making process for gallbladder polyps.

Genetic risk factors

To our knowledge, no research has studied genetic risk factors for gallbladder polyps, despite multiple studies having investigated genetic contributions to gallbladder cancer. For example, studies from Shanghai and Sweden have noted significantly increased risks of gallbladder cancer in patients with a family history of gallbladder cancer[52,53]. It has also been shown in a recent review that approximately one quarter of cases diagnosed in a Utah cohort study were familial[54]. However, the difficulty with evaluating family history as a proxy for genetic factors is that it may also reflect exposure to similar environmental exposures. A recent review has highlighted the paucity of research on specific genetic polymorphisms with respect to gallbladder cancer risk, and extrapolated some biologically plausible hypotheses from gallstone aetiology[54-56]. Overall, there is only low quality evidence for genetic predisposition to gallbladder cancer, and no studies have been conducted for gallbladder polyps. Robust, genome-wide association studies are required to confirm or deny any potential associations.

CONCLUSION

The gaps in the available evidence to support the current guidelines on the management of gallbladder polyps are outlined above. TAUS is the current mainstay for radiological investigation of gallbladder polyps. EUS and HRUS have shown some promise as an adjunct to TAUS but more work is required to assess the exact role and the category of polyps that they may provide diagnostic accuracy. Although polyps of 10 mm and greater are more likely to be true polyps, this cut-off will miss a significant number of true polyps below this threshold and cholecystectomy will also be performed unnecessarily for pseudopolyps when they are greater than 10 mm. The factoring in of the risk factors discussed above to lower the threshold for cholecystectomy will no doubt decrease the number of missed true polyps in the under 10 mm category but cholecystectomy will also be performed when it is not required. No research has been performed to assess the impact of following these guidelines and therefore larger retrospective and prospective case series need to be performed to assess the success of managing gallbladder polyps as per the current guidelines.

REFERENCES

1   **Chattopadhyay D**, Lochan R, Balupuri S, Gopinath BR, Wynne

KS. Outcome of gall bladder polypoidal lesions detected by transabdominal ultrasound scanning: a nine year experience. *World J Gastroenterol* 2005; **11**: 2171-2173 [PMID: 15810087 DOI: 10.3748/wjg.v11.i14.2171]

2   **Andrén-Sandberg A**. Diagnosis and management of gallbladder polyps. *N Am J Med Sci* 2012; **4**: 203-211 [PMID: 22655278 DOI: 10.4103/1947-2714.95897]

3   **Okamoto M**, Okamoto H, Kitahara F, Kobayashi K, Karikome K, Miura K, Matsumoto Y, Fujino MA. Ultrasonographic evidence of association of polyps and stones with gallbladder cancer. *Am J Gastroenterol* 1999; **94**: 446-450 [PMID: 10022644 DOI: 10.1111/j.1572-0241.1999.875_d.x]

4   **Lin WR**, Lin DY, Tai DI, Hsieh SY, Lin CY, Sheen IS, Chiu CT. Prevalence of and risk factors for gallbladder polyps detected by ultrasonography among healthy Chinese: analysis of 34 669 cases. *J Gastroenterol Hepatol* 2008; **23**: 965-969 [PMID: 17725602 DOI: 10.1111/j.1440-1746.2007.05071.x]

5   **Kwon W**, Jang JY, Lee SE, Hwang DW, Kim SW. Clinicopathologic features of polypoid lesions of the gallbladder and risk factors of gallbladder cancer. *J Korean Med Sci* 2009; **24**: 481-487 [PMID: 19543513 DOI: 10.3346/jkms.2009.24.3.481]

6   **Aldridge MC**, Bismuth H. Gallbladder cancer: the polyp-cancer sequence. *Br J Surg* 1990; **77**: 363-364 [PMID: 2187556 DOI: 10.1002/bjs.1800770403]

7   **Kozuka S**, Tsubone N, Yasui A, Hachisuka K. Relation of adenoma to carcinoma in the gallbladder. *Cancer* 1982; **50**: 2226-2234 [PMID: 7127263 DOI: 10.1002/1097-0142(19821115)50:10<2226::AID-CNCR2820501043>3.0.CO;2-3]

8   IARC. Globocan 2012; Available from: URL: http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx

9   World Cancer Research Fund International. Gallbladder cancer statistics. Available from: URL: https://www.wcrf.org/int/cancer-facts-figures/data-specific-cancers/gallbladder-cancer-statistics

10   Cancer Research UK. Cancer Research UK Cancer incidence statistics. 2014; Available from: URL: http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/leukaemia-cll/incidence

11   **Amin MB**, Greene FL, Edge SB. AJCC Cancer Staging Manual. *Sprin Inter Publ*; 2017 [DOI: 10.1007/978-3-319-40618-3]

12   **Keus F**, de Jong JA, Gooszen HG, van Laarhoven CJ. Laparoscopic versus open cholecystectomy for patients with symptomatic cholecystolithiasis. *Cochrane Database Syst Rev* 2006 [PMID: 17054285 DOI: 10.1002/14651858.CD006231]

13   **Lee KF**, Wong J, Li JC, Lai PB. Polypoid lesions of the gallbladder. *Am J Surg* 2004; **188**: 186-190 [PMID: 15249249 DOI: 10.1016/j.amjsurg.2003.11.043]

14   **Gurusamy KS**, Samraj K. Early versus delayed laparoscopic cholecystectomy for acute cholecystitis. Cochrane Database of Systematic Reviews. Vol 67. Chichester, UK: John Wiley & Sons, Ltd; **2006**: 381-387. [PMID 23232475 DOI: 10.1002/14651858.CD005440.pub2]

15   **Krähenbühl L**, Sclabas G, Wente MN, Schäfer M, Schlumpf R, Büchler MW. Incidence, risk factors, and prevention of biliary tract injuries during laparoscopic cholecystectomy in Switzerland. *World J Surg* 2001; **25**: 1325-1330 [PMID: 11596898 DOI: 10.1007/s00268-001-0118-0]

16   **Gurusamy KS**, Abu-Amara M, Farouk M, Davidson BR. Cholecystectomy for gallbladder polyp. *Cochrane Database Syst Rev* 2009 [PMID: 19160315 DOI: 10.1002/14651858.CD007052.pub2]

17   **Christensen M**, Matzen P, Schulze S, Rosenberg J. Complications of ERCP: a prospective study. *Gastrointest Endosc* 2004; **60**: 721-731 [PMID: 15557948 DOI: 10.1016/S0016-5107(04)02169-8]

18   **Matos AS**, Baptista HN, Pinheiro C, Martinho F. [Gallbladder polyps: how should they be treated and when?]. *Rev Assoc Med Bras* (1992) 2010; **56**: 318-321 [PMID: 20676540]

19   **Wiles R**, Thoeni RF, Barbu ST, Vashist YK, Rafaelsen SR, Dewhurst C, Arvanitakis M, Lahaye M, Soltes M, Perinel J, Roberts SA. Management and follow-up of gallbladder polyps: Joint guidelines between the European Society of Gastrointestinal and Abdominal Radiology (ESGAR), European Association for Endoscopic Surgery and other Interventional Techniques (EAES), International Society of Digestive Surgery - European Federation (EFISDS) and European Society of Gastrointestinal Endoscopy (ESGE). *Eur Radiol* 2017; **27**: 3856-3866 [PMID: 28185005 DOI: 10.1007/s00330-017-4742-y]

20   **Gallahan WC**, Conway JD. Diagnosis and management of gallbladder polyps. *Gastroenterol Clin North Am* 2010; **39**: 359-367 [PMID: 20478491 DOI: 10.1016/j.gtc.2010.02.001]

21   **Shapiro RS**, Winsberg F. Comet-tail artifact from cholesterol crystals: observations in the postlithotripsy gallbladder and an in vitro model. *Radiology* 1990; **177**: 153-156 [PMID: 2204960 DOI: 10.1148/radiology.177.1.2204960]

22   **Babu BI**, Dennison AR, Garcea G. Management and diagnosis of gallbladder polyps: a systematic review. *Langenbecks Arch Surg* 2015; **400**: 455-462 [PMID: 25910600 DOI: 10.1007/s00423-015-1302-2]

23   **French DG**, Allen PD, Ellsmere JC. The diagnostic accuracy of transabdominal ultrasonography needs to be considered when managing gallbladder polyps. *Surg Endosc* 2013; **27**: 4021-4025 [PMID: 23749271 DOI: 10.1007/s00464-013-3033-1]

24   **Kim JH**, Lee JY, Baek JH, Eun HW, Kim YJ, Han JK, Choi BI. High-resolution sonography for distinguishing neoplastic gallbladder polyps and staging gallbladder cancer. *AJR Am J Roentgenol* 2015; **204**: W150-W159 [PMID: 25615775 DOI: 10.2214/AJR.13.11992]

25   **Cho JH**, Park JY, Kim YJ, Kim HM, Kim HJ, Hong SP, Park SW, Chung JB, Song SY, Bang S. Hypoechoic foci on EUS are simple and strong predictive factors for neoplastic gallbladder polyps. *Gastrointest Endosc* 2009; **69**: 1244-1250 [PMID: 19249773 DOI: 10.1016/j.gie.2008.10.017]

26   **Jang JY**, Kim SW, Lee SE, Hwang DW, Kim EJ, Lee JY, Kim SJ, Ryu JK, Kim YT. Differential diagnostic and staging accuracies of high resolution ultrasonography, endoscopic ultrasonography, and multidetector computed tomography for gallbladder polypoid lesions and gallbladder cancer. *Ann Surg* 2009; **250**: 943-949 [PMID: 19855259 DOI: 10.1097/SLA.0b013e3181b5d5fc]

27   **Stenberg B**, Elliott S. Diagnosis of gallbladder problems using three-dimensional ultrasound. *Eur Radiol* 2010; **20**: 908-914 [PMID: 19789879 DOI: 10.1007/s00330-009-1614-0]

28   **Numata K**, Oka H, Morimoto M, Sugimori K, Kunisaki R, Nihonmatsu H, Matsuo K, Nagano Y, Nozawa A, Tanaka K. Differential diagnosis of gallbladder diseases with contrast-enhanced harmonic gray scale ultrasonography. *J Ultrasound Med* 2007; **26**: 763-774 [PMID: 17526608 DOI: 10.7863/jum.2007.26.6.763]

29   **Zheng SG**, Xu HX, Liu LN, Lu MD, Xie XY, Wang WP, Hu B, Yan K, Ding H, Tang SS, Qian LX, Luo BM. Contrast-enhanced ultrasound versus conventional ultrasound in the diagnosis of polypoid lesion of gallbladder: a multi-center study of dynamic microvascularization. *Clin Hemorheol Microcirc* 2013; **55**: 359-374 [PMID: 23283444 DOI: 10.3233/CH-121651]

30   **Jenssen C**, Alvarez-Sánchez MV, Napoléon B, Faiss S. Diagnostic endoscopic ultrasonography: assessment of safety and prevention of complications. *World J Gastroenterol* 2012; **18**: 4659-4676 [PMID: 23002335 DOI: 10.3748/wjg.v18.i34.4659]

31   **Sugiyama M**, Xie XY, Atomi Y, Saito M. Differential diagnosis of small polypoid lesions of the gallbladder: the value of endoscopic ultrasonography. *Ann Surg* 1999; **229**: 498-504 [PMID: 10203082 DOI: 10.1016/S0002-9610(00)00526-2]

32   **Cheon YK**, Cho WY, Lee TH, Cho YD, Moon JH, Lee JS, Shim CS. Endoscopic ultrasonography does not differentiate neoplastic from non-neoplastic small gallbladder polyps. *World J Gastroenterol* 2009; **15**: 2361-2366 [PMID: 19452579 DOI: 10.3748/wjg.15.2361]

33   **Choi JH**, Seo DW, Choi JH, Park DH, Lee SS, Lee SK, Kim MH. Utility of contrast-enhanced harmonic EUS in the diagnosis of malignant gallbladder polyps (with videos). *Gastrointest Endosc* 2013; **78**: 484-493 [PMID: 23642490 DOI: 10.1016/j.gie.2013.03.1328]

34   **Kim SY**, Cho JH, Kim EJ, Chung DH, Kim KK, Park YH, Kim YS. The efficacy of real-time colour Doppler flow imaging on endoscopic ultrasonography for differential diagnosis between neoplastic and

non-neoplastic gallbladder polyps. *Eur Radiol* 2018; **28**: 1994-2002 [PMID: 29218621 DOI: 10.1007/s00330-017-5175-3]

35 **Furukawa H**, Kosuge T, Shimada K, Yamamoto J, Kanai Y, Mukai K, Iwata R, Ushio K. Small polypoid lesions of the gallbladder: differential diagnosis and surgical indications by helical computed tomography. *Arch Surg* 1998; **133**: 735-739 [PMID: 9688001 DOI: 10.1001/archsurg.133.7.735]

36 **Lou MW**, Hu WD, Fan Y, Chen JH, E ZS, Yang GF. CT biliary cystoscopy of gallbladder polyps. *World J Gastroenterol* 2004; **10**: 1204-1207 [PMID: 15069726 DOI: 10.3748/wjg.v10.i8.1204]

37 **Irie H**, Kamochi N, Nojiri J, Egashira Y, Sasaguri K, Kudo S. High b-value diffusion-weighted MRI in differentiation between benign and malignant polypoid gallbladder lesions. *Acta Radiol* 2011; **52**: 236-240 [PMID: 21498356 DOI: 10.1258/ar.2010.100234]

38 **Bhatt NR**, Gillis A, Smoothey CO, Awan FN, Ridgway PF. Evidence based management of polyps of the gall bladder: A systematic review of the risk factors of malignancy. *Surgeon* 2016; **14**: 278-286 [PMID: 26825588 DOI: 10.1016/j.surge.2015.12.001]

39 **Park JK**, Yoon YB, Kim YT, Ryu JK, Yoon WJ, Lee SH, Yu SJ, Kang HY, Lee JY, Park MJ. Management strategies for gallbladder polyps: is it possible to predict malignant gallbladder polyps? *Gut Liver* 2008; **2**: 88-94 [PMID: 20485616 DOI: 10.5009/gnl.2008.2.2.88]

40 **Zielinski MD**, Atwell TD, Davis PW, Kendrick ML, Que FG. Comparison of surgically resected polypoid lesions of the gallbladder to their pre-operative ultrasound characteristics. *J Gastrointest Surg* 2009; **13**: 19-25 [PMID: 18972168 DOI: 10.1007/s11605-008-0725-2]

41 **Pedersen MR**, Dam C, Rafaelsen SR. Ultrasound follow-up for gallbladder polyps less than 6 mm may not be necessary. *Dan Med J* 2012; **59**: A4503 [PMID: 23158888 DOI: 10.1148/rg.352140095]

42 **Corwin MT**, Siewert B, Sheiman RG, Kane RA. Incidentally detected gallbladder polyps: is follow-up necessary?--Long-term clinical and US analysis of 346 patients. *Radiology* 2011; **258**: 277-282 [PMID: 20697115 DOI: 10.1148/radiol.10100273]

43 **Lu D**, Radin R, Yung E, Tchelepi H. Malignant transformation of a 5-mm gallbladder polyp over 2 years: a case report and review of current literature. *Ultrasound Q* 2015; **31**: 66-68 [PMID: 25054905 DOI: 10.1097/RUQ.0000000000000094]

44 **Wiles R**, Varadpande M, Muly S, Webb J. Growth rate and malignant potential of small gallbladder polyps--systematic review of evidence. *Surgeon* 2014; **12**: 221-226 [PMID: 24502936 DOI: 10.1016/j.surge.2014.01.003]

45 **Cha BH**, Hwang JH, Lee SH, Kim JE, Cho JY, Kim H, Kim SY. Pre-operative factors that can predict neoplastic polypoid lesions of the gallbladder. *World J Gastroenterol* 2011; **17**: 2216-2222 [PMID: 21633532 DOI: 10.3748/wjg.v17.i17.2216]

46 **Sarkut P**, Kilicturgay S, Ozer A, Ozturk E, Yilmazlar T. Gallbladder polyps: factors affecting surgical decision. *World J Gastroenterol* 2013; **19**: 4526-4530 [PMID: 23901228 DOI: 10.3748/wjg.v19.i28.4526]

47 **Aldouri AQ**, Malik HZ, Waytt J, Khan S, Ranganathan K, Kummaraganti S, Hamilton W, Dexter S, Menon K, Lodge JP, Prasad KR, Toogood GJ. The risk of gallbladder cancer from polyps in a large multiethnic series. *Eur J Surg Oncol* 2009; **35**: 48-51 [PMID: 18339513 DOI: 10.1016/j.ejso.2008.01.036]

48 **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines: management of cholestatic liver diseases. *J Hepatol* 2009; **51**: 237-267 [PMID: 19501929 DOI: 10.1016/j.jhep.2009.04.009]

49 **Said K**, Glaumann H, Bergquist A. Gallbladder disease in patients with primary sclerosing cholangitis. *J Hepatol* 2008; **48**: 598-605 [PMID: 18222013 DOI: 10.1016/j.jhep.2007.11.019]

50 **Leung UC**, Wong PY, Roberts RH, Koea JB. Gall bladder polyps in sclerosing cholangitis: does the 1-cm rule apply? *ANZ J Surg* 2007; **77**: 355-357 [PMID: 17497975 DOI: 10.1111/j.1445-2197.2007.04059.x]

51 **Eaton JE**, Thackeray EW, Lindor KD. Likelihood of malignancy in gallbladder polyps and outcomes following cholecystectomy in primary sclerosing cholangitis. *Am J Gastroenterol* 2012; **107**: 431-439 [PMID: 22031356 DOI: 10.1038/ajg.2011.361]

52 **Hsing AW**, Bai Y, Andreotti G, Rashid A, Deng J, Chen J, Goldstein AM, Han TQ, Shen MC, Fraumeni JF Jr, Gao YT. Family history of gallstones and the risk of biliary tract cancer and gallstones: a population-based study in Shanghai, China. *Int J Cancer* 2007; **121**: 832-838 [PMID: 17450525 DOI: 10.1002/ijc.22756]

53 **Hemminki K**, Li X. Familial liver and gall bladder cancer: a nationwide epidemiological study from Sweden. *Gut* 2003; **52**: 592-596 [PMID: 12631675 DOI: 10.1136/gut.52.4.592]

54 **Liebe R**, Milkiewicz P, Krawczyk M, Bonfrate L, Portincasa P, Krawczyk M. Modifiable Factors and Genetic Predisposition Associated with Gallbladder Cancer. A Concise Review. *J Gastrointestin Liver Dis* 2015; **24**: 339-348 [PMID: 26405706 DOI: 10.15403/jgld.2014.1121.243.lib]

55 **Lee SE**, Jang JY, Lee YJ, Choi DW, Lee WJ, Cho BH, Kim SW; Korean Pancreas Surgery Club. Choledochal cyst and associated malignant tumors in adults: a multicenter survey in South Korea. *Arch Surg* 2011; **146**: 1178-1184 [PMID: 22006877 DOI: 10.1001/archsurg.2011.243]

56 **Buckles DC**, Lindor KD, Larusso NF, Petrovic LM, Gores GJ. In primary sclerosing cholangitis, gallbladder polyps are frequently malignant. *Am J Gastroenterol* 2002; **97**: 1138-1142 [PMID: 12014717 DOI: 10.1111/j.1572-0241.2002.05677.x]

MINIREVIEWS

New horizons in the endoscopic ultrasonography-based diagnosis of pancreatic cystic lesions

María-Victoria Alvarez-Sánchez, Bertrand Napoléon

María-Victoria Alvarez-Sánchez, Instituto de Investigación Sanitaria Galicia Sur, Complejo Hospitalario Universitario de Pontevedra, Pontevedra 36003, Spain

Bertrand Napoléon, Department of Gastroenterology, Ramsay Générale de Santé Private Hospital Jean Mermoz, Lyon 69008, France

ORCID number: María-Victoria Alvarez-Sánchez (0000-0002-2640-2265); Bertrand Napoléon (0000-0001-5866-8132).

Author contributions: Alvarez-Sánchez MV and Napoléon B contributed equally to reviewing the literature, writing and drafting the article, critical revision and final approval.

Correspondence to: María-Victoria Alvarez-Sánchez, MD, Attending Doctor, Department of Gastroenterology, Complejo Hospitalario Universitario de Pontevedra, Instituto de Investigación Sanitaria Galicia Sur (IISGS), Avenida de Mourente sn, Pontevedra 36003, Spain. victoria.alvarez.sanchez@hotmail.com
Telephone: +34-986-800907
Fax: +34-986-800309

Published online: July 14, 2018

Abstract

Pancreatic cystic lesions (PCLs) are increasingly being identified because of the widespread use of high-resolution abdominal imaging. These cysts encompass a spectrum from malignant disease to benign lesions, and therefore, accurate diagnosis is crucial to determine the best management strategy, either surgical resection or surveillance. However, the current standard of diagnosis is not accurate enough due to limitations of imaging and tissue sampling techniques, which entail the risk of unnecessary burdensome surgery for benign lesions or missed opportunities of prophylactic surgery for potentially malignant PCLs. In the last decade, endoscopic innovations based on endoscopic ultrasonography (EUS) imaging have emerged, aiming to overcome the present limitations. These new EUS-based technologies are contrast harmonic EUS, needle-based confocal endomicroscopy, through-the-needle cystoscopy and through-the needle intracystic biopsy. Here, we present a comprehensive and critical review of these emerging endoscopic tools for the diagnosis of PCLs, with a special emphasis on feasibility, safety and diagnostic performance.

Key words: Intraductal papillary mucinous neoplasm; Pancreatic cystic lesions; Endoscopic ultrasonography; Confocal endomicroscopy; Mucinous cystadenoma; Through-the-needle cystoscopy; Serous cystadenoma; Through-the-needle forceps biopsy; Contrast harmonic endoscopic ultrasonography

Core tip: This paper provides a focused update on emerging endoscopic technologies for improving the diagnosis and prediction of the malignant potential of pancreatic cystic lesions. Basic principles, diagnostic

performance, safety and limitations are critically reviewed.

INTRODUCTION

Pancreatic cystic lesions (PCLs), initially thought to be rare, have become an incidental finding increasingly identified because of technological advances and the widespread use of high-resolution abdominal imaging. It is estimated that approximately 3% and 20% of patients undergoing abdominal computed tomography (CT) and magnetic resonance imaging (MRI), respectively, present a PCL[1-2]. Among neoplastic cysts, certain subtypes, basically mucinous cysts, entail a risk of present or future malignancy. Other neoplastic cysts, such as serous cystadenomas (SCAs), are considered benign cysts without potential of malignancy. These two types of neoplastic cysts are sometimes indistinguishable, and therefore, the awareness of possible malignant potential may lead physicians to refer these patients to surgical resection. Despite improvements in pancreatic surgery, considerable morbidity and mortality still occur in 18%-38% and 0.2%-2% of patients who undergo surgery because of PCLs[3-6]. Accurate assessment of malignant potential is therefore of the utmost importance to avoid unnecessary surgery for benign cysts while considering appropriate surveillance for low-risk lesions and surgical treatment for malignant and high-risk cysts.

The initial step of the diagnostic approach to PCLs usually relies on radiological imaging focusing on size and morphological features. Once a PCL has been identified, most commonly by CT, an MRI is recommended due to its higher ability to evaluate nodules and to depict a communication between the cyst and the main pancreatic duct. Owing to the frequent lack of specific radiological features of many PCLs, the overall accuracy of CT and MRI remains low, ranging from 23% to 93%[7-16]. Endoscopic ultrasonography (EUS) further characterizes PCLs and performs better than CT or MRI in assessing the morphology of small cysts and in depicting nodules in mucinous cysts, but EUS imaging alone also shows inadequate accuracy in the range of 40%-94%[17-23]. A major advantage of EUS is the ability to safely obtain cyst fluid for biochemical and cytological analysis. However, the scant cellularity of the cyst fluid accounts for the low diagnostic yield of cytology. Although the cyst fluid carcinoembryonic antigen (CEA) level has been proven to be more accurate than cytology or EUS morphology alone, the reported sensitivity of CEA and cytology for mucinous lesions in a recent meta-analysis

do not exceed 63% and 54%, respectively[24].

All the above limitations have led to the recent updated International, AGA and European guidelines based on predictors of malignancy, which have been shown to be far from providing reliable differentiation between the various PCLs, and recommendations provided in these guidelines are based on low-grade evidence[25-27]. Moreover, the Fukuoka International Consensus Guideline, restricted their recommendations to branch-duct IPMNs that are diagnosed easily in a vast majority of cases (multiple cysts, communication with the main pancreatic duct)[28]. The Fukuoka guidelines were evaluated in a prospective study yielding a high negative predictive value (NPV) of 94% for malignancy but a low positive predictive value (PPV) of approximately 38%, frequently prompting unnecessary surgery[29]. Efforts to overcome current limitations have boosted the research in this area. Molecular analysis of DNA-based biomarkers in cyst fluid has been described with promising results[30]. Genomics, miRNA, proteomics and metabolomics in cyst fluid seem to be promising, but validation studies are pending. In addition to molecular testing, endoscopic innovations based on EUS imaging have emerged in the last decade and they are more easily available than the omics technologies though with a longer learning curve. Although not all of these EUS-based innovations have been validated, they warrant a thorough analysis to evaluate their diagnostic performance and potential impact as a part of the diagnostic workflow of PCLs.

In this article, we aim to review the evolving role of emergent EUS-based technologies-contrast harmonic enhanced imaging, needle-based confocal endomicroscopy, through-the-needle cystoscopy, and through-the-needle intracystic biopsy-in the clinical diagnosis of PCLs, with a critical focus on feasibility, safety and diagnostic performance. Novel approaches to cystic fluid analysis, such as omics technologies and other biomarkers, are beyond of the scope of this review.

A literature search was performed for all available studies concerning the EUS diagnosis of PCLs in PubMed and Embase databases. The following search domains (including closely related words) were used: "pancreatic cysts" in combination with "contrast harmonic EUS" or "contrast enhanced EUS" or "needle confocal endomicroscopy" or "cystoscopy" or "intracystic biopsy". The search was limited to papers published in English until December 2017. Titles were then screened for suitability, and the full-text papers were retrieved. A hand-search of the references listed in the articles accessed was also performed to identify other relevant original studies. Both retrospective and prospective studies reporting data on the feasibility, diagnostic performance and safety of the above referred procedures in patients with PCLs were considered for inclusion. Indications, technical details, performance outcomes, impact on final diagnosis and management, complications and mortality were extracted and further discussed.

CONTRAST-HARMONIC ENHANCED ENDOSCOPIC ULTRASOUND

Because perfusion patterns on CT or MRI explorations allow the characterization of focal lesions, EUS has recently incorporated the use of ultrasound contrast agents (UCAs) to depict blood flow in small vessels. Available UCAs consist of microbubbles composed of an inert gas encapsulated by a shell[31]. Gases are compressible, and when exposed to an ultrasound wave, microbubbles alternatively compress under positive pressure and expand under negative pressure, producing a backscattered acoustic signal with harmonic components[31-32]. These harmonic components are higher than those obtained from tissue and may be selectively detected and reproduced on the ultrasound image for displaying the microvascularity pattern[31]. Not until recently, when new UCAs, broadband EUS transducers and contrast-specific software programs became available, was contrast harmonic EUS (CH-EUS) feasible for the first time, allowing the discrimination between tissue and contrast signals. Second-generation UCAs, which contain a soluble gas, unlike air-filled first-generation agents, are commonly used for CH-EUS. They have a resistant but more flexible and longer lasting shell, making possible the use of low acoustic power (a low mechanical index) and continuous real-time assessment[31-35].

Several steps must be followed to perform CH-EUS[31]. After fundamental B-mode exploration of the target area, a dual screen is displayed, simultaneously showing the CH-EUS image and the conventional B-mode image. Next, optimal parameters, notably, a low MI, should be selected on the ultrasound platform. The UCA is then injected intravenously slowly through a large-gauge intravenous catheter of 16G-18G in order to avoid breaking microbubbles. Finally, the venous catheter must be flushed with saline to clear out persistent microbubbles in the vein. After intravenous injection of microbubbles, it takes 10-20 s to observe the arrival of the contrast agent. The arterial phase lasts 30-45 s, during which the enhancement increases progressively. After the arterial phase, there is a progressive washout of the contrast, and the venous phase persists from 30 s to 120 s.

Clinical outcomes: Review of the literature

Among the 71 articles retrieved from PubMed and Embase databases using the terms "pancreatic cysts" and "contrast harmonic EUS", only seven were suitable for further review[36-42]. All of the studies but one were retrospective, and the majority was devoted to the diagnosis of mural nodules and/or malignancy in intraductal pancreatic mucinous neoplasms (IPMNs). Only three articles addressed the issue of the differential diagnosis of PCLs. Selected articles are summarized in Table 1.

Hocke *et al*[36] performed CH-EUS in 125 patients with PCLs. Contrast enhancement of cyst walls, septa

and nodules was observed in all PCNs, including mucinous cysts, cystic adenocarcinomas, SCAs and cystic neuroendocrine neoplasms (NENs), but in only 6% of nonneoplastic cystic lesions (PCs and dysontogenic cysts). Further supporting these results, in another study by Fusaroli *et al*[37], of 76 patients with PCLs, most SCAs were hyper-enhanced during CH-EUS, without a significant difference (86% *vs* 89%, *P* = NS), when in fact, 90% of pseudocysts showed hypoenhancement (*P* = 0.000004 *vs* serous cysts and *P* = 0.000005 *vs* mucinous cysts). In addition, Kamata *et al*[38] reported that CH-EUS did not add any advantage to EUS for differentiating mucinous and nonmucinous cysts when the presence of mural nodules was considered a sign of mucinous cysts (sensitivity, 79% *vs* 85%; specificity, 96% *vs* 46%; accuracy, 73% *vs* 84%, respectively, *P* = 0.057).

Yamashita *et al*[39] used CT, color Doppler EUS and CH-EUS to prospectively study 17 patients with mural nodules in branch duct type IPMN (BD-IPMN) detected by EUS before being referred for surgery. After pathological analysis, 75% of mural nodules corresponded to adenocarcinomas, and 25% corresponded to adenomas. Compared with surgical specimens, CH-EUS depicted vascularity in all pathologically confirmed nodules and in one case with mucous clots (sensitivity, 100%; specificity, 80%; PPV, 92%; NPV, 100%; and accuracy, 94%). Moreover, the sensitivity of CT and color Doppler-EUS was only 41% and 0%, respectively. Comparable results were found in later studies. Fujita *et al*[40] observed that CT, MRI, and EUS detected mural nodules histologically confirmed in 86%, 71% and 100% of cases. Although EUS was highly sensitive, it was not able to distinguish mucous clots from mural nodules that were correctly classified in all cases after assessing the vascular pattern by CH-EUS (Figure 1). CH-EUS was also shown to be more accurate than CT or EUS in diagnosing mural nodules in the study by Harima *et al*[41] (accuracy of 98%, 72% and 92%, respectively).

Fusaroli *et al*[37] observed that all hyper-enhanced solid components during CH-EUS turned out to be malignant, whereas nonenhanced ones were either mucous clots or internal debris. Nevertheless, other authors found it difficult to discriminate between adenomas or adenocarcinomas in BD-IPMN based on the vascular pattern. Only one study evaluated the accuracy of quantitative CH-EUS for differentiating between low-grade dysplasia (LGD) or intermediate-grade dysplasia and high-grade dysplasia (HGD) or invasive carcinoma. In this study, Yamamoto *et al*[42] retrospectively analyzed the time-intensity curve in 30 patients with resected IPMNs who underwent CH-EUS. The analyzed parameters were the echo intensity change and the echo intensity reduction rate of the mural nodule and the nodule/parenchyma contrast ratio. All of the parameters were significantly higher in the HGD/invasive group (*P* < 0.05), with the nodule/parenchyma contrast ratio being the most accurate parameter (accuracy, 93%). Moreover, a positive linear correlation was observed between the echo intensity change in the mural nodule and the micro-

Authors	Study	*n*	Main outcomes	Complications
Yamashita *et al*[39], 2013	P	17	Differential diagnosis between mural nodules and mucus clots: CH-EUS: Sen 100%, Spe 80%, PPV 92%, NPV 100%, A 94% CT and Doppler-EUS: Sen 41% and 0% respectively	0
Hocke *et al*[36], 2014	R	125	Differential diagnosis between non-neoplastic cysts and PCNs. Hyperenhancement: 100% PCNs Hypoenhancement: 94% non-neoplastic cysts (PCs and dysontogenic cysts) CH-EUS superior to EUS in differential diagnosis between PCNs and non-neoplastic cysts ([a]$P < 0.001$)	0
Harima *et al*[41], 2015	R	30	Performance for diagnosing mural nodules CT: Sen 71%, Spe 100%, PPV 100%, NPV 90%, A 92% EUS: Sen 72%, Spe 61%, PPV 50%, NPV 100%, A 72% CH-EUS: Sen 100%, Spe 97%, PPV 93%, NPV 100%, A 98%	0
Fujita *et al*[40], 2016	R	50	Sensitivity for diagnosing mural nodules: CT: 86% *vs* MRI 71% *vs* EUS 100% EUS was not able to distinguish mural nodules from mucus clots CH-EUS correctly differentiated mural nodules from mucus clots in all cases	0
Fusaroli *et al*[37], 2016	R	76	Differential diagnosis between non-neoplastic cysts and PCNs and between benign and malignant cysts. Hyperenhancement: 86% SCAs and 89% mucinous cysts (P = ns) Hypoenhancement: 90% PCs ([b]$P < 0.000004$ *vs* SCAs and [c]$P < 0.000005$ *vs* mucinous cysts) Hyperenhanced solid components : 100% malignant cysts Non- hype-enhanced solid components: 100% benign cysts	0
Kamata *et al*[38], 2016	R	70	Mural nodule as a sign of mucinous cyst EUS *vs* CH-EUS: Sen 85% *vs* 79%, Spe 46% *vs* 96%, A 73% *vs* 84% ($P = 0.057$) Mural nodule as a sign of malignancy EUS *vs* CH-EUS: Sen 97% *vs* 97%, Spe 40% *vs* 75%, A 64% *vs* 84% ([d]$P = 0.0001$)	0
Yamamoto *et al*[42], 2016	R	30	Quantitative CH-EUS in IPMNs Echo intensity change and echo intensity reduction rate, and nodule/parenchyma contrast ratio significantly higher in HGD/invasive carcinoma ([e]$P < 0.05$) Microvessel density in mural nodule Significantly higher in HGD/invasive carcinoma ([f]$P < 0.002$) Significant correlation between echo intensity change and microvessel density ([g]$P < 0.001$)	0

P: Prospective; R: Retrospective; CT: Computed tomography; MRI: Magnetic resonance imaging; EUS: Endoscopic ultrasonography; CH-EUS: Contrast harmonic endoscopic ultrasonography; Sen: Sensitivity; Spe: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; A: Accuracy; PCNs: Pancreatic cystic neoplasms; IPMN: Intraductal papillary mucinous neoplasm; HGD: High grade dysplasia.

Figure 1 Mural nodule and mucus in branch duct type intraductal pancreatic mucinous neoplasms. A: EUS B mode image. B: Contrast harmonic EUS image. Microbubbles in a mural nodule (green arrow). No bubbles in a mucus clot (orange arrow). EUS: Endoscopic ultrasonography.

vessel density in pathologic specimens (r = 0.803, $P < 0.001$).

No mortality or CH-EUS-related adverse events were reported in any study.

NEEDLE-BASED CONFOCAL LASER ENDOMICROSCOPY

Needle-based confocal laser endomicroscopy (nCLE) is an emergent endoscopic modality that enables imaging of a target tissue at a subcellular level of resolution, providing real-time *in vivo* optical biopsy. For CLE imaging, a low-power laser is used to illuminate the tissue. The laser beam is focused on a plane of interest, and the reflected light from the tissue is filtered and transformed into an electrical signal by a detection system and finally translated into grayscale images by a computer system[43,44]. The final result consists of images with very high spatial resolution and magnification of the focal plane examined within the tissue. Because confocal imaging relies on reflected fluorescent light, intravenous injection of a fluorescent dye, most commonly sodium fluorescein, is required. Fluorescein acts as a contrast agent highlighting blood vessels and tissue architecture[43-46].

Recent advances have allowed the incorporation of CLE technology into a miniprobe of 0.85 mm (AQ-Flex) that can be passed through a 19-gauge (19G) EUS needle. This probe is provided with 10.000 optical fibers and has a field of view of 325 μm, 3.5 μm of lateral resolution and 40-70 μm of confocal depth[46].

Before starting the procedure, a 19G EUS needle is preloaded with the AQ-Flex probe that should be inserted until 2 mm of the probe is positioned beyond the needle tip (Figure 2). At this moment, the probe is fixed to the inlet of the needle channel by a locking system. After identifying the cyst, a single pass with the preloaded 19G

Table 2 Needle-based confocal laser endomicroscopy in pancreatic cystic lesions

Authors	Study	n	Main outcomes	Complications
Konda *et al*[48], 2011	P	16	94% Technical success (feasibility study)	12% post-procedure pancreatitis
Konda *et al*[49], 2013	P	66	Stage 1: Description of visualized structures (*n* = 26) with histological correlation Stage 2: Performance assessment of defined criteria (*n* = 31): Villous pattern: Sen 59%, Spe 100%, PPV 100%, NPV 50%, A 71% for PCNs Significant association with PCNs ([a]*P* = 0.004)	3% post-procedure pancreatitis 4.5% intracystic self-limited bleeding
Nakai *et al*[50], 2015	P	30	Villous pattern in 18 patients with highly certain diagnosis: Sen 80%, Spe 100%, PPV 100%, NPV 80%, A 89% for mucinous cysts Significant association with mucinous cysts ([b]*P* = 0.001)	7% post-procedure (cystoscopy followed by nCLE) pancreatitis
Napoléon *et al*[51], 2015	P	31	Superficial vascular network: Sen 69%, Spe 100%, PPV 100%, NPV 82%, A 87% for SCAs	3% post-procedure pancreatitis
Napoléon *et al*[52], 2016	R	31	Step 1: Description of nCLE patterns for mucinous cysts, PCs and cystic NENs with histological correlation Step 2: Retrospective external validation of nCLE criteria Accuracy 94% for mucinous cysts (90% IPMN - 90% MCA) - 87% SCA - 87% PCs Substantial global IOA: Perfect PC, almost perfect SCA, moderate IPMN, fair MCA	
Kadayifci *et al*[53], 2017	P	20	nCLE performance for mucinous cysts: Sen 66% -Spe 100% -A 80%	0 complications
Krishna *et al*[54], 2017	P	10	Reproducibility of the *in vivo* nCLE criteria in *ex vivo* specimens	
Napoléon *et al*[55], in press	P	209	Diagnostic yield 91% in 78 patients with non-communicating cysts and pathological diagnosis: Sen 95%, Spe 100% - PPV 100% - NPV 98% - A 99% for SCAs Sen 95%, Spe 100% - PPV 100% - NPV 94% - A 97% for mucinous cysts Sen 100%, Spe 95% - PPV 70% - NPV 100% - A 96% for NENs Sen 96%, Spe 95% - PPV 98% - NPV 91% - A R96% for premalignant cysts	1.3% post-procedure pancreatitis
Karia *et al*[56], 2016	R	15	IOA poor to fair for all nCLE variables	
Krishna *et al*[57], 2016	R	49	nCLE performance on 26 patients with definitive diagnosis (23 with pathological diagnosis): Sen 94%, Spe 82% - PPV 88% - NPV 92% - A 89% for mucinous cysts IOA and IOR: Substantial for all nCLE criteria	6.1% post-procedure pancreatitis
Krishna *et al*[58], 2017	R	29	nCLE performance on 29 patients with definitive diagnosis (23 with pathological diagnosis): Sen 95%, Spe 94% - A 95% for mucinous cysts Sen 99%, Spe 98% - A 98% for SCAs Sen 99%, Spe 98% - A 98% for NENs IOA and IOR: Almost perfect for mucinous cysts and SCAs	

P: Prospective; R: Retrospective; Sen: Sensitivity; Spe: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; A: Accuracy; PCNs: Pancreatic cystic neoplasms; PC: Pseudocyst; MCA: Mucinous cystadenoma; SCA: Serous cystadenoma; IPMN: Intraductal papillary mucinous neoplasm; NENs: Neuroendocrine neoplasm; IOA: Interobserver agreement; IOR: Intraobserver reliability.

Figure 2 Needle-based confocal laser endomicroscopy probe in a 19G needle.

EUS needle is performed, and the needle is advanced under EUS guidance until the needle tip contacts the cyst wall. Immediately after, 2.5-5 mL of 10% fluorescein is injected; nCLE imaging begins, during which gentle apposition of the probe to the cyst wall is pursued. The elevator, endoscope dials and torquing are useful for imaging in a fanning technique[43]. Because images are obtained at a rate of 12 frames/s, video-recording is always performed for 2-5 min and further reviewed with a dedicated software program. At the end of the procedure, the probe is withdrawn, and the cyst fluid is aspirated as per standard practice. Antibiotic prophylaxis is systematically administered[47].

Clinical outcomes: Review of the literature
A literature search in PubMed and Embase databases identified 24 articles reporting on nCLE in PCLs. Only 11 of these articles were deemed eligible and consisted of 7 prospective and 4 retrospective studies (Table 2)[48-58]. Most of them were focused on feasibility, safety and performance in differentiating mucinous from nonmucinous cysts or in the differential diagnosis among the

Figure 3 Needle-based confocal laser endomicroscopy image of an intraductal pancreatic mucinous neoplasms displaying multiple papillary projections.

Figure 4 Needle-based confocal laser endomicroscopy image of the superficial vascular network pattern of a serous cystadenoma. Multiple interconnected vessels (green arrows). Red cells inside displayed as black structures (orange arrow).

Figure 5 Staining with a vascular marker of serous cystadenomas histological specimen. Capillary necklace with subepithelial vessels showed in brown.

several types of PCLs. Three studies aimed to externally validate the nCLE criteria, and one study performed an *ex vivo* validation of the *in vivo* nCLE criteria.

The first series was reported in 2011 by Konda *et al*[48] and was a feasibility study of 16 cysts and 2 solid masses of the pancreas. The nCLE procedure was feasible in 15 of 16 cysts, although the technical challenges described

in 6 of them were related to the transduodenal approach, the post loading technique (the insertion of the CLE probe after positioning the EUS needle inside the lesion) and the longer length of the metallic tip at the distal end of the probe. Complications occurred in two patients, both of whom developed pancreatitis requiring hospitalization. This study was followed by a larger multicenter study (*in vivo* nCLE Study in the Pancreas with Endosonography of Cystic Tumors, INSPECT) that was reported by the same group and that aimed to evaluate the diagnostic potential and safety of nCLE in the differential diagnosis of PCLs in 66 patients[49]. A consensus description of visualized structures on 26 patients was achieved, and the correlation between histology and nCLE was investigated during the first stage of the study. Then, the performance of nCLE criteria to identify PCNs, including mucinous cystadenoma (MCA), IPMN or adenocarcinoma, was assessed in 31 additional patients. The presence of villous structures was highly specific (100%) but provided low sensitivity, at 59%, and it was the only specific finding having a significant association with PCNs ($P = 0.004$). Post procedure pancreatitis occurred in 3% of cases: one patient experienced transient abdominal pain, and three cases of intracystic bleeding were observed and spontaneously solved.

The DETECT trial (Diagnosis of Pancreatic Cysts: Endoscopic Ultrasound, Through-the-Needle Confocal Laser Endomicroscopy and Cystoscopy Trial) was designed to evaluate the diagnostic yield of cystoscopy followed by nCLE in 30 patients[50]. A highly certain diagnosis was possible in 18 of these patients based on the clinical presentation, other image findings, fluid analysis and cytology. In these patients, a papillary projection (Figure 3) and/or a dark ring on nCLE, corresponding to the villous pattern previously reported by Konda, was associated with mucinous cysts ($P = 0.001$) with 80% sensitivity, 100% specificity, 100% PPV, 80% NPV and 89% accuracy. Two patients (7%) developed post procedure pancreatitis.

After the above initial experiences, Napoléon and colleagues described new nCLE criteria based on histological correlation in two consecutive studies[51-52]. In the first one, a criterion for *in vivo* diagnosis of SCA was defined and consensually identified as a superficial vascular network (Figure 4). The presence of small and regular structures circulating inside the opacified channels during nCLE suggested the vascular nature of these channels, which was confirmed by histological assessment of surgical specimens (Figure 5). This vascular network was demonstrated to be at a superficial depth of 50-70 μm and, therefore, at the reach of the nCLE probe. Moreover, SCA was the only PCL that featured this pattern among 31 patients with PCLs of unknown diagnosis. The sensitivity, specificity, PPV, NPV and accuracy of this criterion for diagnosing SCA were 69%, 100%, 100%, 82% and 87%, respectively. Only one patient suffered mild acute pancreatitis (3%). In a second study (CONTACT 1), the same authors identified three other nCLE criteria that included a thick gray line

Figure 6 Epithelial border image in a mucinous cystadenoma at needle-based confocal laser endomicroscopy.

Figure 7 Heterogeneous sized grey and white particles in a pseudocyst at needle-based confocal laser endomicroscopy.

for mucinous neoplasms (Figure 6), a field of bright particles for PCs (Figure 7) and black neoplastic clusters with white fibrous areas for NENs. In this case, the histological correlation was the epithelial border, a mix of inflammatory cells and neoplastic cell proliferation with several forms of architectural organization respectively. In the retrospective validation, four external and blinded reviewers evaluated the diagnostic performance of these criteria. A conclusive diagnosis was achieved in 23 of 31 patients (74%). Overall, the accuracy of nCLE criteria was 94% for mucinous cysts, 87% for SCA and 87% for PC. Trends toward high specificity were also shown (> 90% for mucinous cysts and 100% for nonmucinous cysts). NENs were excluded from the external validation due to the small number in this series (*n* = 2). More recently, Krishna *et al*[54] have further confirmed the reproducibility of the *in vivo* nCLE criteria in *ex vivo* specimens of 10 patients with surgically resected PCLs.

To overcome the limitations of the reduced number of patients and the lack of pathological confirmation in previous series, Napoléon *et al*[55] designed a larger multicenter and prospective study (CONTACT 2) whose results are now available (submitted). Among 209 enrolled patients with a noncommunicating solitary cyst, 78 patients with a final diagnosis proven by surgical histopathology or cytopathological analysis of cyst fluid by EUS fine needle aspiration (EUS-FNA) were included

in the final results. The overall diagnostic yield of nCLE was 91%, and the sensitivity and specificity for the main types of PCLs were higher than 95%. Perfect specificity (100%) was observed for diagnosing SCA and premalignant mucinous cysts. Furthermore, the nCLE area under the curve was significantly higher than that of CEA dosage for differentiating mucinous from nonmucinous cysts ($P < 0.01$) and higher than that of EUS morphology in differentiating between premalignant and benign PCLs ($P < 0.05$). However, nCLE criteria were not highly specific for NENs and PCs. NEN criteria were also observed in one cystic solid pseudopapillary neoplasia (CSPPN), one cystic lymphoma and 1 PC. Additionally, one mucinous lesion and one SCA exhibited PC criteria. Acute pancreatitis occurred only in 1.3% of patients. Although the impact of nCLE on patient management was not evaluated, it is noteworthy that in 29% of patients with a previous inconclusive EUS-FNA, nCLE was conclusive in 91% of cases and 100% accurate.

The first reports on interobserver agreement (IOA) yielded diverging results[51,56]. In the first one (CONTACT 1), four external reviewers assessed the IOA of the nCLE criteria in 31 cases[51]. The diagnostic accuracy for mucinous cysts was 94%, and the global IOA was rated as substantial (k = 0.72, 95%CI: 0.52-0.87). A later validation study reported low diagnostic accuracy for the type of PCL (46%), with an IOA ranging from poor to fair for all nCLE variables[56]. However, careful interpretation of these results is advised due to limitations such as poor image quality and short duration of video capture, which may have accounted for the poor results, and other methodological issues such as the lack of intraobserver reliability and few patients with a histological gold-standard diagnosis. More recently, two other studies aimed to validate nCLE criteria. One investigation consisted of a retrospective analysis at a single center[57,58]. nCLE videos from 26 patients (23 with pathological diagnosis) were reviewed by 6 blinded nCLE-naïve observers[57]. Substantial IOA and IOR were achieved for differentiating mucinous from nonmucinous cysts (k = 0.67, 95%CI: 0.57-0.77 and k = 0.78 ± 0.13, respectively) and for detecting all nCLE criteria. These results were further corroborated by an international external interobserver and intraobserver study[58]. In 29 patients, the overall accuracy of nCLE for the diagnosis of mucinous cysts was 95%, with an almost perfect IOA and IOR among six expert endosonographers with nCLE experience (k = 0.81, 95%CI: 0.71-0.90 and k = 0.86 ± 0.11, respectively). Furthermore, nCLE was 98% accurate in diagnosing SCA and the IOA and IOR for recognizing the fern pattern (previously defined as superficial vascular network) were also almost perfect (k = 0.83, 95%CI: 0.73-0.92 and k = 0.85 ± 0.11, respectively).

THROUGH-THE-NEEDLE CYSTOSCOPY

Through-the-needle cystoscopy is a procedure that allows

Figure 8 Moray forceps.

direct assessment of the cyst content as well as the inner cyst wall by means of single-operator cholangioscopy fiberoptic probe (Spyglass®, Boston Scientific, Natick, Mass, United States). The probe has 6000-pixel optic bundles, a 300 cm working length and a diameter of 0.77 mm. It provides a 70-degree field of view and has a 2-7 mm focal length[43,50]. The cystic cavity is accessed under EUS guidance with a 19G EUS needle. Before starting the procedure, the needle stylet is removed, and the fiberoptic probe is preloaded through the 19G needle and prefitted with the advancement of the probe 2 mm beyond the needle tip. Then, the probe is withdrawn 2-3 mm inside the needle, and once inside the cyst, the probe is advanced again to the prefitted position. Prophylactic antibiotics are always given, and cystoscopy images may be recorded for further review[50].

Clinical outcomes: Review of the literature
Of the 8 references retrieved, only 3 suitable articles were identified[50,59-60]. The first one reported on two patients who underwent through-the-needle cystoscopy followed by biliary forceps biopsy[59]. In both cases, it was possible to rule out a pseudocyst because a flat normal mucosa was visualized lining the inner cyst wall. In addition, cystoscopy enabled a better delineation of mural nodules and targeted biopsies of the selected suspicious areas. Severe acute pancreatitis occurred in one patient one month after the procedure. It is not possible to rule out a delayed procedure-related complication, although very unlikely.

In the prospective DETECT study, Nakai *et al*[50] performed through-the needle-cystoscopy followed by nCLE in 30 patients. The cyst content was evaluated for clarity, the presence of mucin or debris, and the smoothness, nodularity and vascularity of the cyst walls were assessed. The median image time of cystoscopy was 4 min, and 33% of the images were rated as fair or poor. The mucinous content was described as viscous and cloudy fluid. Typical findings and their clinical correlations were finger-like projections and a mucin cloud in IPMNs, smooth cyst walls with cloudy fluid in MCNs, and smooth cyst walls with prominent regular vessels in SCAs. However, the only significant association in 18 high-certainty diagnoses was observed between mucin on cystoscopy and mucinous cysts ($P = 0.0004$), with 90% sensitivity, 100% specificity, 100% PPV, 100% NPV and 94% accuracy. However, finger-like projections were identified in only two of the ten high-certainty mucinous lesions. When cystoscopy and nCLE were combined, the sensitivity increased from 90% to 100%. Post procedure pancreatitis was reported twice.

In a retrospective study published last year, Chai *et al*[60] performed through-the-needle cystoscopy in 43 patients. Based on the blood vessel distribution, the presence of partitions or ridge-like structures and the presence of papilla-like structures, the characteristic findings of different PCLs were defined and then validated by surgical pathology, FNA or fluid cytology. The authors concluded that a tree-like branching pattern of blood vessels may suggest the diagnosis of SCA (specificity, 91%; sensitivity, 69%) and that intracystic papilla-like structures may be characteristic of mucinous cysts (specificity, 92%; sensitivity, 22%). No pancreatitis was observed, and only two patients presented mild abdominal post procedure pain.

THROUGH-THE-NEEDLE FORCEPS BIOPSY
The low sensitivity of EUS-FNA cytology because of relatively acellular samples makes appealing the possibility to obtain biopsies. The design of minibiopsy forceps has led to the development of a new EUS-FNA tissue acquisition technique. Moray® micro forceps (US Endoscopy, Ohio, United States) were designed for use in EUS procedures to enhance sampling from lesions that can occur within and outside the gastrointestinal tract, leading to a more definitive diagnosis (Figure 8). These forceps are 230 cm in length and have serrated jaws (jaw opening of 4.3 mm) and a spring sheath 0.8 mm in diameter, allowing use through a 19G EUS needle[43,61].

Clinical outcomes: Review of the literature
No formal study was retrieved after searching PubMed and Embase for "pancreatic cyst" and "intracystic biopsy". Only two pilot studies reporting on 2 cases each and four additional case reports were identified[59,61-65].

Through-the-needle intracystic biopsy was first described by Aparicio *et al*[59] in two patients. They used 0.8 mm endoscopic retrograde cholangiopancreatography biopsy forces followed by a 3-4 min observation with a fiberoptic probe to rule out immediate bleeding. In both cases, a mucinous-like cylindric epithelium without cellular atypia was observed. As stated above, one patient presented severe acute pancreatitis one month later. After this preliminary experience, six more cases were reported to undergo intracystic biopsy, enabling the correct diagnosis of 5 mucinous cysts (one of them with mild dysplasia) and one benign lymphoepithelial cyst without any complication[61-65].

DISCUSSION
PCLs remain a diagnostic and therapeutic challenge to clinicians. Several issues remain to be solved, notably, how to improve diagnosis and better predict malignant behavior. It has been reported that 36% of SCAs are treated with unnecessary surgery because of an uncertain diagnosis[66]. In the attempt to cover this need,

Figure 9 Moray forceps inside a cyst. Green arrow: The tip of the 19G needle. Orange arrow: The tip of the Moray forceps grasping the cyst wall.

several EUS-based tools have emerged recently and some experience is now available using these technologies in the diagnosis of PCLs.

On the grounds that CH-EUS has been proved to be accurate for the differential diagnosis of solid pancreatic masses, it was hypothesized that CH-EUS might also be helpful in PCLs, and some experience has been reported in the last five years[67-70]. Due to its high spatial resolution, CH-EUS may define the inner structure of cysts by depicting small septa or mural nodules that become echogenic during CH-EUS, whereas the intracystic content remains invisible (Figure 9). This modality may assist not only in the differential diagnosis but also in identifying malignancy risk features.

Current evidence suggests that CH-EUS is highly accurate for distinguishing nonneoplastic cysts (PCs and dysontogenic cysts) from neoplastic cysts because the former do not exhibit cystic wall vascularization. This feature prevents pointless and onerous surgery in this benign setting. However, a different scenario is observed among different neoplastic cysts whose biological behavior may be significantly different and where misinterpretations are common in CH-EUS. Benign SCAs, the most common nonmucinous cystic neoplasms, and potentially malignant mucinous cysts show undistinctive features on CH-EUS. Consequently CH-EUS cannot be used for the differential diagnosis of neoplastic cysts.

The presence of mural nodules is considered in the international consensus guidelines on IPMN management as a high-risk stigma and strongly supports surgical resection. Mural nodules may sometimes be too small for detection by CT or MRI. The high spatial resolution of EUS enables better identification in these cases. However, the performance of EUS is not enough to discriminate between mural nodules or mucous clots. Hyperenhancement of solid components during CH-EUS may differentiate mural nodules from mucous clots or debris. This step is essential to avoid unnecessary surgery and is included in the last guidelines[25]. Nodules in BD-IPMNs include not only malignant nodules but also benign adenomas that might be followed without surgery, but the preoperative differentiation between them does not seem possible with qualitative CH-EUS. Angiogenesis plays a key role in tumor growth and progression; in

fact, neovascularization was reported to be crucial in the tumorigenesis of invasive IPMNs with a progressive increase in microvessel density from benign to malignant tissue[71]. According to this observation, preliminary experience with quantitative CH-EUS suggests that the analysis of echo intensity changes during CH-EUS may be an accurate method to discriminate between low/intermediate-grade dysplasia and high-grade dysplasia/invasive carcinoma. Further trials are necessary to confirm this interest.

When it comes to safety, CH-EUS exhibits an excellent safety profile with no adverse events in series reporting on PCLs. UCAs are the sole factor adding risk to conventional EUS. Concerns were raised after a study reporting on UCAs used for stress echocardiography, where 4 deaths and 190 serious adverse events were observed and associated with the use of UCAs[72]. Consequently, the Food and Drug Administration issued a black box warning regarding the use of UCAs in several pathologic cardiorespiratory states. Later experience in large cohorts of patients has shown that the rate of UCA-related serious adverse events is lower than 0.01%, with anaphylactoid reactions occurring in 1/10.000 cases[73-74].

More recent evidence confirms that nCLE is feasible during EUS-FNA and allows *in vivo* diagnosis of PCLs with high accuracy; nCLE criteria have been defined for IPMN, MCA, SCA, PC and cystic NEN with a proven histopathological correlation. Unlike CH-EUS, a perfect specificity of 100% has been confirmed with nCLE for benign SCA and for mucinous lesions. The superficial vascular network is exclusive to SCAs, allowing SCA diagnosis with high confidence, therefore preventing unnecessary surgical resection. Sensitivity of this pattern, although high, is not perfect, and epithelium denudation may account for the lack of a vascular pattern in oligocystic SCA. Specificity is also perfect for the overall group of mucinous lesions, but when they are further classified as either IPMN or MCA, the specific findings of papillae or epithelial borders, respectively, may both be present. Other times, papillae or epithelial borders cannot be visualized, and the explanation is the nonuniform distribution of papillae throughout the epithelium of IPMNs or modifications induced by inflammation in some MCAs. These inflammatory changes are also the reason why a field of bright, gray and black particles is not specific for PCs and may be found in other cystic tumors following infection or bleeding. Finally, the presence of dark spots of cell aggregates surrounded by gray areas of fibrosis and vessels, initially attributed to NENs, is not specific and may be present in other premalignant lesions, such as CSPPNs. However, due to the similar premalignant nature, the impact on clinical management of misdiagnosis between NENs and CSPPNs or between IPMNs and MCAs is of little importance.

The major concern related to nCLE is the risk of acute pancreatitis, which has ranged from 1.3% in the largest and most recent study to 12% in the first feasibility study. The use of Spyglass® (Boston Scientific, Natick, Mass, United States) for cystoscopy may also explain

the second highest rate of pancreatitis in the DETECT study[50]. The average rate from the rest of the studies is 2.7%, which is comparable with that of the conventional EUS-FNA procedure[75]. Only one series reported intracystic bleeding in 3 patients (4.5%); however, no intervention was required, and they were solved spontaneously. Several factors that add to the risk of complications have been suggested, such as needle size, duration of the procedure and abrasion of the cyst lining with the needle tip. Some tips have been suggested during nCLE to maximize the procedure safety. The interposition of the main pancreatic duct should be avoided when selecting the puncture site to lower the risk of acute pancreatitis. Once inside the cyst, limited brushing of the cyst wall is recommended to avoid cystic bleeding and pancreatitis; instead, it is preferred to perform consecutive apposition between the probe and the cyst wall. Finally, the exploration should be stopped as soon as nCLE criteria are met, or after 6 min if no specific criteria are found, to minimize the risk of adverse events[43]. No study reported adverse events related to the injection of fluorescein, but severe allergic reactions are possible, although very uncommon (1/222000)[76]. Minor side effects, such as nausea, vomiting, mild epigastric pain, transient hypotension, injection site erythema or diffuse rash, have been reported in 1.4% of cases[77]. The learning curve for nCLE seems to have an important effect on the pancreatitis rate since one study reported three acute-pancreatitis cases among the first 25 patients and none in the successive 34 patients[58]. Technical challenges reported in initial feasibility studies are now overcome, and several tips have been suggested to avoid them (preloading of the nCLE probe, use of the most flexible needle). However, other limitations remain to be solved. The most important one is the limited surface of the cystic wall that is accessible to be scanned. Exploration is feasible over the area in front of the needle tip but less than 50% of the cystic surface is likely to be visualized. The second argued limitation is the high cost of the probe, which is considered a limiting step to implementing nCLE technology in routine practice. Nevertheless, a health economic evaluation carried out in France demonstrated that nCLE resulted in a reduction of 23% of surgical interventions[78]. This finding translated into a reduction in clinical costs of 13% in the public sector and 14% in the private sector. The improved diagnostic accuracy of nCLE reduces the number of false positives and false negatives, avoiding unnecessary surgical interventions and lifelong surveillance for benign cysts.

The heterogeneous distribution of neoplastic tissue in PCLs makes it reasonable to attempt to directly explore the inner cyst walls. Limited experience with through-the-needle cystoscopy has demonstrated the feasibility for direct visualization of cyst walls and has suggested that it may help to target biopsies to suspicious areas. Patterns at cystoscopy have been proposed for SCAs, MCNs and IPMNs but have not been validated yet. Like for nCLE, the main complication is

acute pancreatitis, and therefore, limiting the time inside the cyst is strongly recommended. The greatest interest seems to be the ability to detect mucin clots that have a typical appearance on cystoscopy. Nevertheless, the performance of cystoscopy was not higher than the string test that is more simple and free of cost[50]. Beside the cost of the fiberoptic system, other limitations of cystoscopy in reported studies are mainly related to the suboptimal quality of images. Therefore, the real place of cystoscopy need to be established.

Through-the-needle intracystic biopsy has been suggested to be a feasible technique for tissue acquisition in one series and several case reports. It allows histological diagnosis including the grade of dysplasia, although the retrieved samples are small. However, the processing of the samples is not always easy. Because of the small-sized specimens, they sometimes disintegrate during fixation in formalin. In addition, lesions such as mural nodules may not be targetable by the stiff 19G EUS-FNA, especially those located in the uncinate process. Finally, concerns remain about the risk of bleeding following through-the-needle forceps biopsy, similarly to brush cytology, even if no cases of bleeding have been reported. Formal studies in larger series are required to validate the results of this new technique and to confirm its safety.

In summary, CH-EUS seems to be a safe and complementary tool to EUS-FNA for the assessment of PCLs. CH-EUS is especially accurate in differentiating nonneoplastic cysts from PCNs and mural nodules from mucus clots. Moreover, the recent International Association of Pancreatology and the European guidelines have recommended CH-EUS for further evaluation of mural nodules in IPMN and PCN respectively. Larger and prospective studies are required to confirm the role of quantitative CH-EUS in the differential diagnosis between malignant and benign mucinous neoplasms. EUS nCLE is a minimally invasive tool with remarkable potential for diagnosing PCLS. Future research should also address new nCLE criteria associated with the grade of dysplasia or cancer, the additional clinical value of combining nCLE with the current standard of PCLs diagnosis and the cost-effectiveness of nCLE during the initial EUS-FNA or after inconclusive results of EUS-FNA. Meanwhile, and based on available evidence at present, excluding cost-effectiveness, we propose an algorithm of diagnosis for PCLs (Figure 10). Through-the-needle cystoscopy and biopsy must be evaluated in formal studies in larger series to validate their results and to confirm their safety before integrating them in the diagnostic flowchart of PCLs.

CONCLUSION

PCLs are increasingly identified on imaging, but their characterization remains challenging due to limitations of the current endoscopic and imaging techniques. In this article, we presented a comprehensive review about emerging endoscopic tools for the diagnosis of PCLs.

Figure 10 Diagnostic algorithm integrating new endoscopic ultrasonography-based technologies. PCL: Pancreatic cystic lesión; PC: Pseudocyst; MPD: Main pancreatic cyst; BD-IPMN: Branch duct intraductal pancreatic mucinous neoplasm; MCN: Mucinous cystic neoplasm; SCA: Serous cystadenoma; NEN: Neuroendocrine tumour; CT: Computed tomography, MRI: Magnetic resonance imaging; EUS: Endoscopic ultrasonography; CH-EUS: Contrast harmonic enhanced EUS; EUS-FNA: EUS guided fine needle aspiration; nCLE: Needle-based confocal endomicroscopy.

Among them, through-the-needle cystoscopy and biopsy still have the lowest amounts of available evidence, with the most extensive experience reported for CH-EUS and nCLE. Both modalities have been demonstrated to provide valuable information for the decision-making process and to be supplementary techniques to EUS-FNA. Limitations for their widespread implementation are their elevated cost and learning curve. Future studies should address their clinical impact on patient management, the optimal timing for their application in the diagnostic work flow of PCLs and their cost-effectiveness. Through-the-needle cystoscopy and intracystic biopsy must be further evaluated in formal and larger trials. Moreover, because a combination of these new techniques may further improve our ability to diagnose PCLs, multi-arm trials incorporating these new technologies and emergent molecular markers would be of most value to determine the best diagnostic approach.

REFERENCES

1 **Laffan TA**, Horton KM, Klein AP, Berlanstein B, Siegelman SS, Kawamoto S, Johnson PT, Fishman EK, Hruban RH. Prevalence of unsuspected pancreatic cysts on MDCT. *AJR Am J Roentgenol* 2008; **191**: 802-807 [PMID: 18716113 DOI: 10.2214/AJR.07.3340]

2 **Lee KS**, Sekhar A, Rofsky NM, Pedrosa I. Prevalence of incidental pancreatic cysts in the adult population on MR imaging. *Am J Gastroenterol* 2010; **105**: 2079-2084 [PMID: 20354507 DOI: 10.1038/ajg.2010.122]

3 **Ferrone CR**, Correa-Gallego C, Warshaw AL, Brugge WR, Forcione DG, Thayer SP, Fernández-del Castillo C. Current trends in pancreatic cystic neoplasms. *Arch Surg* 2009; **144**: 448-454 [PMID: 19451487 DOI: 10.1001/archsurg.2009.36]

4 **Valsangkar NP**, Morales-Oyarvide V, Thayer SP, Ferrone CR, Wargo JA, Warshaw AL, Fernández-del Castillo C. 851 resected cystic tumors of the pancreas: a 33-year experience at the Massachusetts General Hospital. *Surgery* 2012; **152**: S4-S12 [PMID: 22770958 DOI: 10.1016/j.surg.2012.05.033]

5 **Salvia R**, Fernández-del Castillo C, Bassi C, Thayer SP, Falconi M, Mantovani W, Pederzoli P, Warshaw AL. Main-duct intraductal papillary mucinous neoplasms of the pancreas: clinical predictors of malignancy and long-term survival following resection. *Ann Surg* 2004; **239**: 678-685; discussion 685-687 [PMID: 15082972 DOI: 10.1097/01.sla.0000124386.54496.15]

6 **Sohn TA**, Yeo CJ, Cameron JL, Hruban RH, Fukushima N, Campbell KA, Lillemoe KD. Intraductal papillary mucinous neoplasms of the pancreas: an updated experience. *Ann Surg* 2004; **239**: 788-797; discussion 797-799 [PMID: 15166958 DOI: 10.1097/01.sla.0000128306.90650.aa]

7 **Procacci C**, Biasiutti C, Carbognin G, Accordini S, Bicego E, Guarise A, Spoto E, Andreis IA, De Marco R, Megibow AJ. Characterization of cystic tumors of the pancreas: CT accuracy. *J Comput Assist Tomogr* 1999; **23**: 906-912 [PMID: 10589565 DOI: 10.1097/00004728-199911000-00014]

8 **Curry CA**, Eng J, Horton KM, Urban B, Siegelman S, Kuszyk BS, Fishman EK. CT of primary cystic pancreatic neoplasms: can CT be used for patient triage and treatment? *AJR Am J Roentgenol* 2000; **175**: 99-103 [PMID: 10882255 DOI: 10.2214/ajr.175.1.1750099]

9 **Visser BC**, Yeh BM, Qayyum A, Way LW, McCulloch CE, Coakley FV. Characterization of cystic pancreatic masses: relative accuracy of CT and MRI. *AJR Am J Roentgenol* 2007; **189**: 648-656 [PMID: 17715113 DOI: 10.2214/AJR.07.2365]

10 **Chaudhari VV**, Raman SS, Vuong NL, Zimmerman P, Farrell J, Reber H, Sayre J, Lu DS. Pancreatic cystic lesions: discrimination accuracy based on clinical data and high resolution CT features. *J Comput Assist Tomogr* 2007; **31**: 860-867 [PMID: 18043347 DOI: 10.1097/RCT.0b013e318039b277]

11 **Lu X**, Zhang S, Ma C, Peng C, Lv Y, Zou X. The diagnostic value of EUS in pancreatic cystic neoplasms compared with CT and MRI. *Endosc Ultrasound* 2015; **4**: 324-329 [PMID: 26643701 DOI: 10.4103/2303-9027.170425]

12   **Sahani DV**, Sainani NI, Blake MA, Crippa S, Mino-Kenudson M, del-Castillo CF. Prospective evaluation of reader performance on MDCT in characterization of cystic pancreatic lesions and prediction of cyst biologic aggressiveness. *AJR Am J Roentgenol* 2011; **197**: W53-W61 [PMID: 21700995 DOI: 10.2214/AJR.10.5866]

13   **Sainani NI**, Saokar A, Deshpande V, Fernández-del Castillo C, Hahn P, Sahani DV. Comparative performance of MDCT and MRI with MR cholangiopancreatography in characterizing small pancreatic cysts. *AJR Am J Roentgenol* 2009; **193**: 722-731 [PMID: 19696285 DOI: 10.2214/AJR.08.1253]

14   **de Jong K**, van Hooft JE, Nio CY, Gouma DJ, Dijkgraaf MG, Bruno MJ, Fockens P. Accuracy of preoperative workup in a prospective series of surgically resected cystic pancreatic lesions. *Scand J Gastroenterol* 2012; **47**: 1056-1063 [PMID: 22571417 DOI: 10.310 9/00365521.2012.674970]

15   **Fisher WE**, Hodges SE, Yagnik V, Morón FE, Wu MF, Hilsenbeck SG, Raijman IL, Brunicardi FC. Accuracy of CT in predicting malignant potential of cystic pancreatic neoplasms. *HPB* (Oxford) 2008; **10**: 483-490 [PMID: 19088937 DOI: 10.1080/136518208022 91225]

16   **Lee HJ**, Kim MJ, Choi JY, Hong HS, Kim KA. Relative accuracy of CT and MRI in the differentiation of benign from malignant pancreatic cystic lesions. *Clin Radiol* 2011; **66**: 315-321 [PMID: 21356393 DOI: 10.1016/j.crad.2010.06.019]

17   **Brugge WR**, Lewandrowski K, Lee-Lewandrowski E, Centeno BA, Szydlo T, Regan S, del Castillo CF, Warshaw AL. Diagnosis of pancreatic cystic neoplasms: a report of the cooperative pancreatic cyst study. *Gastroenterology* 2004; **126**: 1330-1336 [PMID: 15131794 DOI: 10.1053/j.gastro.2004.02.013]

18   **Sedlack R**, Affi A, Vazquez-Sequeiros E, Norton ID, Clain JE, Wiersema MJ. Utility of EUS in the evaluation of cystic pancreatic lesions. *Gastrointest Endosc* 2002; **56**: 543-547 [PMID: 12297771 DOI: 10.1067/mge.2002.128106]

19   **Stojanović N**, Ruvidić R, Jovčić G, Mijović A. Drug-induced agranulocytosis: bone marrow granulocytic progenitor cells. *Biomed Pharmacother* 1990; **44**: 181-184 [PMID: 2397279 DOI: 10.1067/mge.2002.128542]

20   **Pais SA**, Attasaranya S, Leblanc JK, Sherman S, Schmidt CM, DeWitt J. Role of endoscopic ultrasound in the diagnosis of intraductal papillary mucinous neoplasms: correlation with surgical histopathology. *Clin Gastroenterol Hepatol* 2007; **5**: 489-495 [PMID: 17350894 DOI: 10.1016/j.cgh.2006.12.007]

21   **Koito K**, Namieno T, Nagakawa T, Shyonai T, Hirokawa N, Morita K. Solitary cystic tumor of the pancreas: EUS-pathologic correlation. *Gastrointest Endosc* 1997; **45**: 268-276 [PMID: 9087833 DOI: 10.1016/S0016-5107(97)70269-4]

22   **Gerke H**, Jaffe TA, Mitchell RM, Byrne MF, Stiffler HL, Branch MS, Baillie J, Jowell PS. Endoscopic ultrasound and computer tomography are inaccurate methods of classifying cystic pancreatic lesions. *Dig Liver Dis* 2006; **38**: 39-44 [PMID: 16314152 DOI: 10.1016/j.dld.2005.09.023]

23   **Ahmad NA**, Kochman ML, Brensinger C, Brugge WR, Faigel DO, Gress FG, Kimmey MB, Nickl NJ, Savides TJ, Wallace MB, Wiersema MJ, Ginsberg GG. Interobserver agreement among endosonographers for the diagnosis of neoplastic versus non-neoplastic pancreatic cystic lesions. *Gastrointest Endosc* 2003; **58**: 59-64 [PMID: 12838222 DOI: 10.1067/mge.2003.298]

24   **Thornton GD**, McPhail MJ, Nayagam S, Hewitt MJ, Vlavianos P, Monahan KJ. Endoscopic ultrasound guided fine needle aspiration for the diagnosis of pancreatic cystic neoplasms: a meta-analysis. *Pancreatology* 2013; **13**: 48-57 [PMID: 23395570 DOI: 10.1016/j.pan.2012.11.313]

25   **Takaori K**. "Revisions of the International Consensus Fukuoka Guidelines for the Management of IPMN of the Pancreas": Progress for twelve years. *Pancreatology* 2017; **17**: 645-646 [PMID: 28864411 DOI: 10.1016/j.pan.2017.08.008]

26   **Vege SS**, Ziring B, Jain R, Moayyedi P; Clinical Guidelines Committee; American Gastroenterology Association. American gastroenterological association institute guideline on the diagnosis and management of asymptomatic neoplastic pancreatic cysts.

Gastroenterology 2015; **148**: 819-822; quize12-13 [PMID: 25805375 DOI: 10.1053/j.gastro.2015.01.015]

27   **European Study Group on Cystic Tumours of the Pancreas.** European evidence-based guidelines on pancreatic cystic neoplasms. *Gut* 2018; **67**: 789-804 [PMID: 29574408 DOI: 10.1136/gutjnl-2018-316027]

28   **Tanaka M**, Fernández-del Castillo C, Adsay V, Chari S, Falconi M, Jang JY, Kimura W, Levy P, Pitman MB, Schmidt CM, Shimizu M, Wolfgang CL, Yamaguchi K, Yamao K; International Association of Pancreatology. International consensus guidelines 2012 for the management of IPMN and MCN of the pancreas. *Pancreatology* 2012; **12**: 183-197 [PMID: 22687371 DOI: 10.1016/j.pan.2012.04.004]

29   **Robles EP**, Maire F, Cros J, Vullierme MP, Rebours V, Sauvanet A, Aubert A, Dokmak S, Lévy P, Ruszniewski P. Accuracy of 2012 International Consensus Guidelines for the prediction of malignancy of branch-duct intraductal papillary mucinous neoplasms of the pancreas. *United European Gastroenterol J* 2016; **4**: 580-586 [PMID: 27536368 DOI: 10.1177/2050640615623370]

30   **Springer S**, Wang Y, Dal Molin M, Masica DL, Jiao Y, Kinde I, Blackford A, Raman SP, Wolfgang CL, Tomita T, Niknafs N, Douville C, Ptak J, Dobbyn L, Allen PJ, Klimstra DS, Schattner MA, Schmidt CM, Yip-Schneider M, Cummings OW, Brand RE, Zeh HJ, Singhi AD, Scarpa A, Salvia R, Malleo G, Zamboni G, Falconi M, Jang JY, Kim SW, Kwon W, Hong SM, Song KB, Kim SC, Swan N, Murphy J, Geoghegan J, Brugge W, Fernandez-Del Castillo C, Mino-Kenudson M, Schulick R, Edil BH, Adsay V, Paulino J, van Hooft J, Yachida S, Nara S, Hiraoka N, Yamao K, Hijioka S, van der Merwe S, Goggins M, Canto MI, Ahuja N, Hirose K, Makary M, Weiss MJ, Cameron J, Pittman M, Eshleman JR, Diaz LA Jr, Papadopoulos N, Kinzler KW, Karchin R, Hruban RH, Vogelstein B, Lennon AM. A combination of molecular markers and clinical features improve the classification of pancreatic cysts. *Gastroenterology* 2015; **149**: 1501-1510 [PMID: 26253305 DOI: 10.1053/j.gastro.2015.07.041]

31   **Alvarez-Sánchez MV**, Napoléon B. Contrast-enhanced harmonic endoscopic ultrasound imaging: basic principles, present situation and future perspectives. *World J Gastroenterol* 2014; **20**: 15549-15563 [PMID: 25400439 DOI: 10.3748/wjg.v20.i42.15549]

32   **Ignee A**, Atkinson NS, Schuessler G, Dietrich CF. Ultrasound contrast agents. *Endosc Ultrasound* 2016; **5**: 355-362 [PMID: 27824024 DOI: 10.4103/2303-9027.193594]

33   **Kang ST**, Yeh CK. Ultrasound microbubble contrast agents for diagnostic and therapeutic applications: current status and future design. *Chang Gung Med J* 2012; **35**: 125-139 [PMID: 22537927]

34   **Kitano M**, Kudo M, Sakamoto H, Komaki T. Endoscopic ultrasonography and contrast-enhanced endoscopic ultrasonography. *Pancreatology* 2011; **11** Suppl 2: 28-33 [PMID: 21464584 DOI: 10.1159/000323493]

35   **Sanchez MV**, Varadarajulu S, Napoleon B. EUS contrast agents: what is available, how do they work, and are they effective? *Gastrointest Endosc* 2009; **69**: S71-S77 [PMID: 19179175 DOI: 10.1016/j.gie.2008.12.004]

36   **Hocke M**, Cui XW, Domagk D, Ignee A, Dietrich CF. Pancreatic cystic lesions: The value of contrast-enhanced endoscopic ultrasound to influence the clinical pathway. *Endosc Ultrasound* 2014; **3**: 123-130 [PMID: 24955342 DOI: 10.4103/2303-9027.131040]

37   **Fusaroli P**, Serrani M, De Giorgio R, D'Ercole MC, Ceroni L, Lisotti A, Caletti G. Contrast Harmonic-Endoscopic Ultrasound Is Useful to Identify Neoplastic Features of Pancreatic Cysts (With Videos). *Pancreas* 2016; **45**: 265-268 [PMID: 26474428 DOI: 10.1097/MPA.0000000000000441]

38   **Kamata K**, Kitano M, Omoto S, Kadosaka K, Miyata T, Yamao K, Imai H, Sakamoto H, Harwani Y, Chikugo T, Chiba Y, Matsumoto I, Takeyama Y, Kudo M. Contrast-enhanced harmonic endoscopic ultrasonography for differential diagnosis of pancreatic cysts. *Endoscopy* 2016; **48**: 35-41 [PMID: 26605974 DOI: 10.1055/s-0034-1393564]

39   **Yamashita Y**, Ueda K, Itonaga M, Yoshida T, Maeda H, Maekita T, Iguchi M, Tamai H, Ichinose M, Kato J. Usefulness of contrast-enhanced endoscopic sonography for discriminating mural nodules

from mucous clots in intraductal papillary mucinous neoplasms: a single-center prospective study. *J Ultrasound Med* 2013; **32**: 61-68 [PMID: 23269711 DOI: 10.7863/jum.2013.32.1.61]

40 **Fujita M**, Itoi T, Ikeuchi N, Sofuni A, Tsuchiya T, Ishii K, Kamada K, Umeda J, Tanaka R, Tonozuka R, Honjo M, Mukai S, Moriyasu F. Effectiveness of contrast-enhanced endoscopic ultrasound for detecting mural nodules in intraductal papillary mucinous neoplasm of the pancreas and for making therapeutic decisions. *Endosc Ultrasound* 2016; **5**: 377-383 [PMID: 28000629 DOI: 10.4103/2303 -9027.190927]

41 **Harima H**, Kaino S, Shinoda S, Kawano M, Suenaga S, Sakaida I. Differential diagnosis of benign and malignant branch duct intraductal papillary mucinous neoplasm using contrast-enhanced endoscopic ultrasonography. *World J Gastroenterol* 2015; **21**: 6252-6260 [PMID: 26034360 DOI: 10.3748/wjg.v21.i20.6252]

42 **Yamamoto N**, Kato H, Tomoda T, Matsumoto K, Sakakihara I, Noma Y, Horiguchi S, Harada R, Tsutsumi K, Hori K, Tanaka T, Okada H, de Yamamoto K. Contrast-enhanced harmonic endoscopic ultrasonography with time-intensity curve analysis for intraductal papillary mucinous neoplasms of the pancreas. *Endoscopy* 2016; **48**: 26-34 [PMID: 26561919 DOI: 10.1055/s-0034-1393563]

43 **Leung Ki EL**, Napoleon B. Nuevas herramientas y recursos en la evaluación de quistes pancreáticos por ultrasonografía endoscópica.In: Fauze Maluf-Filho/Shyam Varadarajulu. Avances en ultrasonografía endoscópica. *Clínicas iberoamericanas de gastroenterología y hepatología* 2018; 51-68

44 **Nakai Y**, Isayama H, Shinoura S, Iwashita T, Samarasena JB, Chang KJ, Koike K. Confocal laser endomicroscopy in gastrointestinal and pancreatobiliary diseases. *Dig Endosc* 2014; **26** Suppl 1: 86-94 [PMID: 24033351 DOI: 10.1111/den.12152]

45 **Giovannini M**. Needle-based confocal laser endomicroscopy. *Endosc Ultrasound* 2015; **4**: 284-288 [PMID: 26643694 DOI: 10.41 03/2303-9027.170405]

46 **Tsujino T**, Yan-Lin Huang J, Nakai Y, Samarasena JB, Lee JG, Chang KJ. In vivo identification of pancreatic cystic neoplasms with needle-based confocal laser endomicroscopy. *Best Pract Res Clin Gastroenterol* 2015; **29**: 601-610 [PMID: 26381305 DOI: 10.1016/ j.bpg.2015.06.006]

47 **Ştefănescu D**, Pereira SP, Keane M, Săftoiu A. Needle-based confocal laser endomicroscopy in pancreatic cystic tumors assessment. *Rom J Morphol Embryol* 2015; **56**: 1263-1268 [PMID: 26743270]

48 **Konda VJ**, Aslanian HR, Wallace MB, Siddiqui UD, Hart J, Waxman I. First assessment of needle-based confocal laser endomicroscopy during EUS-FNA procedures of the pancreas (with videos). *Gastrointest Endosc* 2011; **74**: 1049-1060 [PMID: 21924718 DOI: 10.1016/j.gie.2011.07.018]

49 **Wefers H**, Sies H. Generation of photoemissive species during quinone redox cycling. *Biochem Pharmacol* 1986; **35**: 22-24 [PMID: 2416319 DOI: 10.1055/s-0033-1344714]

50 **Nakai Y**, Iwashita T, Park DH, Samarasena JB, Lee JG, Chang KJ. Diagnosis of pancreatic cysts: EUS-guided, through-the-needle confocal laser-induced endomicroscopy and cystoscopy trial: DETECT study. *Gastrointest Endosc* 2015; **81**: 1204-1214 [PMID: 25634486 DOI: 10.1016/j.gie.2014.10.025]

51 **Napoléon B**, Lemaistre AI, Pujol B, Caillol F, Lucidarme D, Bourdariat R, Morellon-Mialhe B, Fumex F, Lefort C, Lepilliez V, Palazzo L, Monges G, Filoche B, Giovannini M. A novel approach to the diagnosis of pancreatic serous cystadenoma: needle-based confocal laser endomicroscopy. *Endoscopy* 2015; **47**: 26-32 [PMID: 25325684 DOI: 10.1055/s-0034-1390693]

52 **Napoleon B**, Lemaistre AI, Pujol B, Caillol F, Lucidarme D, Bourdariat R, Morellon-Mialhe B, Fumex F, Lefort C, Lepilliez V, Palazzo L, Monges G, Poizat F, Giovannini M. In vivo characterization of pancreatic cystic lesions by needle-based confocal laser endomicroscopy (nCLE): proposition of a comprehensive nCLE classification confirmed by an external retrospective evaluation. *Surg Endosc* 2016; **30**: 2603-2612 [PMID: 26428198 DOI: 10.1007/s00464-015-4510-5]

53 **Kadayifci A**, Atar M, Basar O, Forcione DG, Brugge WR. Needle-Based Confocal Laser Endomicroscopy for Evaluation of Cystic Neoplasms of the Pancreas. *Dig Dis Sci* 2017; **62**: 1346-1353 [PMID: 28281172 DOI: 10.1007/s10620-017-4521-2]

54 **Krishna SG**, Modi RM, Kamboj AK, Swanson BJ, Hart PA, Dillhoff ME, Manilchuk A, Schmidt CR, Conwell DL. In vivo and ex vivo confocal endomicroscopy of pancreatic cystic lesions: A prospective study. *World J Gastroenterol* 2017; **23**: 3338-3348 [PMID: 28566895 DOI: 10.3748/wjg.v23.i18.3338]

55 **Napoléon B**, Palazzo M, Lemaistre AI, Caillol F, Palazzo L, Aubert A, Buscail L, Maire F, Mialhe-Morellon B, Pujol B, Giovannini M. Needle-based confocal laser endomicroscopy characterization of pancreatic cystic lesions with a definitive diagnoses: a prospective multicentre study. *Endoscopy* 2017; In press

56 **Karia K**, Waxman I, Konda VJ, Gress FG, Sethi A, Siddiqui UD, Sharaiha RZ, Kedia P, Jamal-Kabani A, Gaidhane M, Kahaleh M. Needle-based confocal endomicroscopy for pancreatic cysts: the current agreement in interpretation. *Gastrointest Endosc* 2016; **83**: 924-927 [PMID: 26382051 DOI: 10.1016/j.gie.2015.08.080]

57 **Krishna SG**, Swanson B, Hart PA, El-Dika S, Walker JP, McCarthy ST, Malli A, Shah ZK, Conwell DL. Validation of diagnostic characteristics of needle based confocal laser endomicroscopy in differentiation of pancreatic cystic lesions. *Endosc Int Open* 2016; **4**: E1124-E1135 [PMID: 27853737 DOI: 10.1055/s-0042-116491]

58 **Krishna SG**, Brugge WR, Dewitt JM, Kongkam P, Napoleon B, Robles-Medranda C, Tan D, El-Dika S, McCarthy S, Walker J, Dillhoff ME, Manilchuk A, Schmidt C, Swanson B, Shah ZK, Hart PA, Conwell DL. Needle-based confocal laser endomicroscopy for the diagnosis of pancreatic cystic lesions: an international external interobserver and intraobserver study (with videos). *Gastrointest Endosc* 2017; **86**: 644-654.e2 [PMID: 28286093 DOI: 10.1016/ j.gie.2017.03.002]

59 **Aparicio JR**, Martínez J, Niveiro M, Cabezas A, Ruiz F, De Madaria E, Casellas JA. Direct intracystic biopsy and pancreatic cystoscopy through a 19-gauge needle EUS (with videos). *Gastrointest Endosc* 2010; **72**: 1285-1288 [PMID: 20970789 DOI: 10.1016/j.gie.2010.08.036]

60 **Chai N**, Feng J, Guo Y, Li H, Ning B, Wang X, Wang Y, Wang Y, Zhai Y, Linghu E. Preliminary study of single-operator cholangioscopy for diagnosing pancreatic cystic lesions. *Gastrointest Endosc* 2017; **86**: 208-218 [PMID: 28185905 DOI: 10.1016/ j.gie.2017.01.038]

61 **Shakhatreh MH**, Naini SR, Brijbassie AA, Grider DJ, Shen P, Yeaton P. Use of a novel through-the-needle biopsy forceps in endoscopic ultrasound. *Endosc Int Open* 2016; **4**: E439-E442 [PMID: 27092324 DOI: 10.1055/s-0042-101941]

62 **Barresi L**, Tarantino I, Ligresti D, Curcio G, Granata A, Traina M. A new tissue acquisition technique in pancreatic cystic neoplasm: endoscopic ultrasound-guided through-the-needle forceps biopsy. *Endoscopy* 2015; **47** Suppl 1: E297-E298 [PMID: 26099102 DOI: 10.1055/s-0034-1392031]

63 **Pham KD**, Engjom T, Gjelberg Kollesete H, Helgeland L. Diagnosis of a mucinous pancreatic cyst and resection of an intracystic nodule using a novel through-the-needle micro forceps. *Endoscopy* 2016; **48** Suppl 1: E125-E126 [PMID: 27031299 DOI: 10.1055/ s-0042-105437]

64 **Attili F**, Pagliari D, Rimbas M, Inzani F, Brizi MG, Costamagna G, Larghi A. Endoscopic ultrasound-guided histological diagnosis of a mucinous non-neoplastic pancreatic cyst using a specially designed through-the-needle microforceps. *Endoscopy* 2016; **48** Suppl 1: E188-E189 [PMID: 27213974 DOI: 10.1055/s-0042-108194]

65 **Huelsen A**, Cooper C, Saad N, Gupta S. Endoscopic ultrasound-guided, through-the-needle forceps biopsy in the assessment of an incidental large pancreatic cystic lesion with prior inconclusive fine-needle aspiration. *Endoscopy* 2017; **49**: E109-E110 [PMID: 28192812 DOI: 10.1055/s-0043-100217]

66 **Jais B**, Rebours V, Malleo G, Salvia R, Fontana M, Maggino L, Bassi C, Manfredi R, Moran R, Lennon AM, Zaheer A, Wolfgang C, Hruban R, Marchegiani G, Fernández Del Castillo C, Brugge W, Ha Y, Kim MH, Oh D, Hirai I, Kimura W, Jang JY, Kim SW, Jung W, Kang H, Song SY, Kang CM, Lee WJ, Crippa S, Falconi

M, Gomatos I, Neoptolemos J, Milanetto AC, Sperti C, Ricci C, Casadei R, Bissolati M, Balzano G, Frigerio I, Girelli R, Delhaye M, Bernier B, Wang H, Jang KT, Song DH, Huggett MT, Oppong KW, Pererva L, Kopchak KV, Del Chiaro M, Segersvard R, Lee LS, Conwell D, Osvaldt A, Campos V, Aguero Garcete G, Napoleon B, Matsumoto I, Shinzeki M, Bolado F, Fernandez JM, Keane MG, Pereira SP, Acuna IA, Vaquero EC, Angiolini MR, Zerbi A, Tang J, Leong RW, Faccinetto A, Morana G, Petrone MC, Arcidiacono PG, Moon JH, Choi HJ, Gill RS, Pavey D, Ouaïssi M, Sastre B, Spandre M, De Angelis CG, Rios-Vives MA, Concepcion-Martin M, Ikeura T, Okazaki K, Frulloni L, Messina O, Lévy P. Serous cystic neoplasm of the pancreas: a multinational study of 2622 patients under the auspices of the International Association of Pancreatology and European Pancreatic Club (European Study Group on Cystic Tumors of the Pancreas). *Gut* 2016; **65**: 305-312 [PMID: 26045140 DOI: 10.1136/gutjnl-2015-309638]

67   **Kitano M**, Kudo M, Yamao K, Takagi T, Sakamoto H, Komaki T, Kamata K, Imai H, Chiba Y, Okada M, Murakami T, Takeyama Y. Characterization of small solid tumors in the pancreas: the value of contrast-enhanced harmonic endoscopic ultrasonography. *Am J Gastroenterol* 2012; **107**: 303-310 [PMID: 22008892 DOI: 10.1038/ajg.2011.354]

68   **Napoleon B**, Alvarez-Sanchez MV, Gincoul R, Pujol B, Lefort C, Lepilliez V, Labadie M, Souquet JC, Queneau PE, Scoazec JY, Chayvialle JA, Ponchon T. Contrast-enhanced harmonic endoscopic ultrasound in solid lesions of the pancreas: results of a pilot study. *Endoscopy* 2010; **42**: 564-570 [PMID: 20593334 DOI: 10.1055/s-0030-1255537]

69   **Gincul R**, Palazzo M, Pujol B, Tubach F, Palazzo L, Lefort C, Fumex F, Lombard A, Ribeiro D, Fabre M, Hervieu V, Labadie M, Ponchon T, Napoléon B. Contrast-harmonic endoscopic ultrasound for the diagnosis of pancreatic adenocarcinoma: a prospective multicenter trial. *Endoscopy* 2014; **46**: 373-379 [PMID: 24532350 DOI: 10.1055/s-0034-1364969]

70   **Omoto S**, Takenaka M, Kitano M, Miyata T, Kamata K, Minaga K, Arizumi T, Yamao K, Imai H, Sakamoto H, Harwani Y, Sakurai T, Watanabe T, Nishida N, Takeyama Y, Chiba Y, Kudo M. Characterization of Pancreatic Tumors with Quantitative Perfusion Analysis in Contrast-Enhanced Harmonic Endoscopic Ultrasonography. *Oncology* 2017; **93** Suppl 1: 55-60 [PMID: 29258065 DOI: 10.1159/000481231]

71   **Tachezy M**, Reichelt U, Melenberg T, Gebauer F, Izbicki JR, Kaifi JT. Angiogenesis index CD105 (endoglin)/CD31 (PECAM-1) as a predictive factor for invasion and proliferation in intraductal papillary mucinous neoplasm (IPMN) of the pancreas. *Histol Histopathol* 2010; **25**: 1239-1246 [PMID: 20712008 DOI: 10.14670/HH-25.1239]

72   **Main ML**. Ultrasound contrast agent safety: from anecdote to evidence. *JACC Cardiovasc Imaging* 2009; **2**: 1057-1059 [PMID: 19761982 DOI: 10.1016/j.jcmg.2009.05.008]

73   **Szebeni J**. Complement activation-related pseudoallergy: a new class of drug-induced acute immune toxicity. *Toxicology* 2005; **216**: 106-121 [PMID: 16140450 DOI: 10.1016/j.tox.2005.07.023]

74   **Piscaglia F**, Bolondi L; Italian Society for Ultrasound in Medicine and Biology (SIUMB) Study Group on Ultrasound Contrast Agents. The safety of Sonovue in abdominal applications: retrospective analysis of 23188 investigations. *Ultrasound Med Biol* 2006; **32**: 1369-1375 [PMID: 16965977 DOI: 10.1016/j.ultrasmedbio.2006.05.031]

75   **de Jong K**, Poley JW, van Hooft JE, Visser M, Bruno MJ, Fockens P. Endoscopic ultrasound-guided fine-needle aspiration of pancreatic cystic lesions provides inadequate material for cytology and laboratory analysis: initial results from a prospective study. *Endoscopy* 2011; **43**: 585-590 [PMID: 21611945 DOI: 10.1055/s-0030-1256440]

76   **Yannuzzi LA**, Rohrer KT, Tindel LJ, Sobel RS, Costanza MA, Shields W, Zang E. Fluorescein angiography complication survey. *Ophthalmology* 1986; **93**: 611-617 [PMID: 3523356 DOI: 10.1016/S0161-6420(86)33697-2]

77   **Wallace MB**, Meining A, Canto MI, Fockens P, Miehlke S, Roesch T, Lightdale CJ, Pohl H, Carr-Locke D, Löhr M, Coron E, Filoche B, Giovannini M, Moreau J, Schmidt C, Kiesslich R. The safety of intravenous fluorescein for confocal laser endomicroscopy in the gastrointestinal tract. *Aliment Pharmacol Ther* 2010; **31**: 548-552 [PMID: 20002025 DOI: 10.1111/j.1365-2036.2009.04207.x]

78   **Le Pen C**, Palazzo L, Napoléon B. A health economic evaluation of needle-based confocal laser endomicroscopy for the diagnosis of pancreatic cysts. *Endosc Int Open* 2017; **5**: E987-E995 [PMID: 29159273 DOI: 10.1055/s-0043-117947]

ORIGINAL ARTICLE

Basic Study

Total polysaccharides of the Sijunzi decoction attenuate tumor necrosis factor-α-induced damage to the barrier function of a Caco-2 cell monolayer *via* the nuclear factor-κB-myosin light chain kinase-myosin light chain pathway

Yue Lu, Leng Li, Jin-Wei Zhang, Xiao-Qin Zhong, Jian-An Wei, Ling Han

Yue Lu, Leng Li, Jin-Wei Zhang, Xiao-Qin Zhong, Jian-An Wei, Ling Han, The Second Clinical College (Guangdong Provincial Hospital of Chinese Medicine), Guangzhou University of Chinese Medicine, Guangzhou 510006, Guangdong Province, China

ORCID number: Yue Lu (0000-0002-5340-8398); Leng Li (0000-0002-2011-7527); Jin-Wei Zhang (0000-0003-2886-3222); Xiao-Qin Zhong (0000-0002-2673-493X); Jian-An Wei (0000-0003-1556-1321); Ling Han (0000-0002-6168-8828).

Abstract

AIM
To explore the protective effects and underlying mechanisms of total polysaccharides of the Sijunzi decoction (TPSJ) on the epithelial barriers *in vitro*.

METHODS
Caco-2 cell monolayers were treated with or without TPSJ in the presence or absence of TNF-α, and paracellular permeability and transepithelial electrical resistance (TEER) were measured to evaluate the epithelial barrier function. Immunofluorescence and western blotting were respectively used to evaluate the distribution and expression of the tight junction proteins claudin 1, claudin 2, zo3, and occludin in Caco-2 cells. Western blotting was also used to evaluate the cellular expression of myosin light chain (MLC), phosphorylated MLC (pMLC), MLC kinase (MLCK), and nuclear factor (NF)-κB p65.

RESULTS
TPSJ promoted the proliferation of Caco-2 cells and inhibited TNF-α-induced secretion of pro-inflammatory cytokines. Furthermore, TPSJ significantly ameliorated both

the reduction of TEER and the increased paracellular permeability observed in tumor necrosis factor (TNF)-α-damaged Caco-2 monolayers. Furthermore, TPSJ remarkably attenuated TNF-α-induced morphological changes, downregulated the expression of claudin 1, claudin 2, zo3, and occludin, and markedly suppressed TNF-α-mediated upregulation of p-MLC and MLCK expression. Finally, TPSJ inhibited the activation and expression of NF-κB p65.

CONCLUSION
Our results demonstrate that TPSJ alleviates the TNF-α-induced impairment of the intestinal epithelial cell barrier function by suppressing NF-κB p65-mediated phosphorylation of MLCK and MLC.

Key words: Inflammatory bowel disease; Tight junction; Total polysaccharides of the Sijunzi decoction; Nuclear factor-κB pathway

Core tip: Total polysaccharides of the Sijunzi decoction (TPSJ) comprise the active ingredient of Sijunzi decoction, which has long been used to treat gastrointestinal tract disorders. However, the mechanisms by which TPSJ affects the intestinal epithelial barrier remain unclear. Our study results demonstrated that TPSJ attenuated tumor necrosis factor (TNF)-α-induced intestinal barrier dysfunction in a Caco-2 cell monolayer. Furthermore, TPSJ inhibited TNF-α-induced upregulation of myosin light chain (MLC) phosphorylation, which is mediated by MLC kinase and NF-κB, suggesting that this mechanism might underlie the protective effects of TPSJ against intestinal epithelial barrier dysfunction triggered by proinflammatory cytokines.

INTRODUCTION

The intestinal epithelial barrier plays a crucial role in separating luminal microbes and antigenic molecules from the internal milieu. Accordingly, intestinal barrier dysfunction can destroy immune homeostasis and induce an inflammatory response[1,2]. Tight junctions (TJs), which are mediated by proteins such as claudins, occludin, and zonula occludens, are necessary for epithelial barrier maintenance[3-5]. Disruption of the intestinal epithelial barrier can increase intestinal permeability, a crucial pathogenic contributor to intestinal inflammation[6,7].

Intestinal epithelial barrier disruption is common to many inflammatory enteropathies, including Crohn's disease (CD), ulcerative colitis (UC), and infectious diarrhea[8-11].

Many locally released pro-inflammatory cytokines and mediators, including tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1β, Th17 type cytokines (IL-17, IL-23, IL-22, and IL-6), and nitric oxide (NO) have been recognized to contribute to intestinal barrier dysfunction during the course of inflammatory bowel disease *in vitro* and *in vivo*[12-17]. Additionally, myosin light chain kinase (MLCK), which mediates the phosphorylation of myosin light chain (MLC), is thought to play a critical role in proinflammatory cytokine-induced intestinal barrier disruption[18,19].

Sijunzi decoction (SJZD) is a traditional medicinal formula comprising four Chinese herbs: *Ginseng Radix et Rhizoma* or *Codonopsispilosula, Atractylodes Macrocephalae Rhizoma*, *Poria*, and *Glycyrrhizae Radix et Rhizoma Praeparatecum Melle*. This decoction, which is used to strengthen the spleen and tonify the qi, has been used to treat gastrointestinal tract diseases since ancient times[20-22]. In a previous study, SJZD was shown to regulate both digestive system and immune system function[23]. In mice, total polysaccharides of the Sijunzi decoction (TPSJ) were shown to antagonize cyclophosphamide-induced injury to the intestinal mucosal associated lymphoid tissues[24], suggesting that these polysaccharides could improve intestinal mucosal immune function. Another study found that polysaccharides of the SJZD can restore intestinal function and protect against indomethacin-induced damage to IEC-6 rat intestinal epithelial cells[25].

Our previous studies suggested that TPSJ could inhibit the proliferation of IEC-6 cells *in vitro*. However, no reports have discussed the ability of TPSJ to regulate the intestinal epithelial barrier. In the present study, we explored the effects of TPSJ on the intestinal barrier formed by Caco-2 human colon adenocarcinoma cells damaged by the proinflammatory cytokine TNF-α, as well as the underlying mechanism. Our results indicate that TPSJ could relieve the intestinal epithelial barrier dysfunction induced by TNF-α, and that this function was mediated by the downregulation of MLCK-dependent MLC phosphorylation in a manner dependent on nuclear factor (NF)-κB p65.

MATERIALS AND METHODS

Plant materials
The Sijunzi Decoction (SJZD) used in this research comprised *Ginseng Radix et Rhizoma* or *Codonopsispilosula, Atractylodes Macrocephalae Rhizoma*, *Poria* and *Glycyrrhizae Radix et Rhizoma Praeparatecum Melle*. These four drugs were pharmacopoeia-grade. All herbs were obtained from Kangmei Pharmaceutical Company Ltd. (Guangzhou, Guangdong, China).

Reagents
Dulbecco's modified Eagle's medium (DMEM), non-

Figure 1 Gel permeation chromatography of total polysaccharides of the Sijunzi decoction.

essential amino acids (NEAA), and fetal bovine serum (FBS) were obtained from GIBCO Laboratories (Grand Island, NY, United States). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and phenolsulfonphthalein were obtained from Sigma-Aldrich (St. Louis, MO, United States). Fluoroisothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) were purchased from Lianke Biotechnology Co (Hangzhou, China). Antibodies specific for claudin 1, claudin 2, zo3, occludin, MLC (phospho S20), MLC (pan), and MLCK and the NF-κB p50/p65 transcription factor assay kit were all purchased from Abcam (Cambridge, MA, United States). Enzyme-linked immunosorbent (ELISA) kits for TNF-α, IL-6 and IL-8 were obtained from eBioscience (San Diego, CA, United States).

Preparation of TPSJ

SJZD comprised *Ginseng Radix et Rhizoma* or *Codonopsispilosula, Atractylodes Macrocephalae Rhizoma*, *Poria*, and *Glycyrrhizae Radix et Rhizoma Praeparatecum Melle* at a ratio of 3:3:3:2 to yield a total weight of 1100 g. All of the herbs were placed in a container to which a volume of cold water approximately 12 times (7.2 L) the solid volume was added. After soaking the herbs for 2 h, we boiled the mixture for 30 min and filtered the herbs, reserving the filtrate. Subsequently, we added another volume of water approximately 5 times the volume of herbs to the container and boiled the mixture for 30 min, followed by filtration. We then mixed the two filtrates and concentrated the liquid to 1.4 L. Subsequently, we added ethanol to the filtrates to yield an alcohol concentration of 75% and stored them at 4 ℃ overnight. The next day, we filtered, precipitated, and dissolved the ethanol mixture in approximately 1.6 L of ultrapure water, followed by centrifugation at 8400 rpm for 15 min. The resulting supernatant was frozen and dried to yield the total polysaccharide. A phenol-sulfuric acid spectrophotometry method was used to measure the polysaccharide content (as glucose), which was 70.61% $\pm$ 1.70%, according to at least three independent experiments. Figure 1 depicts the gel permeation chromatography (GPC) analysis of TPSJ[26].

Cell culture

Caco-2 human colon adenocarcinoma cells were ob-

tained from the Cell Culture Unit of Shanghai Science Academy (Shanghai, China). The cells were grown in DMEM supplemented with 10% FBS and 1% NEAA and incubated in a humidified atmosphere with 5% CO_2 atmosphere at 37 ℃.

MTT assay

Cell viability was determined using a MTT reduction assay. Cells were seeded into 96-well plates in DMEM + 10% FBS + 1% NEAA at a density of 5000 per well and treated with 100 ng/mL TNF-α. After a 24-h incubation, TPSJ or DMEM (control) was added to the wells, followed by another 24-h incubation. Subsequently, 10 μL of MTT solution was added to each well, and the plates were incubated for 4 h. Finally, we lysed the cells with 0.04 N HCl in isopropyl alcohol and read the absorbance of each well at 570 nm.

Flow cytometric quantification of apoptosis

To assess apoptosis, we harvested Caco-2 cells. After two washes with phosphate-buffered saline (PBS), we resuspended the cells in 200 μL of Annexin-V binding buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 2 mmol/L $MgCl_2$, 5 mmol/L KCl, 2.5 mmol/L $CaCl_2$, pH 7.4) and added 10 μL of FITC-conjugated Annexin V to each tube according to the manufacturer's protocol. Following a 15 min incubation in the dark at room temperature, we added 10 μL of PI and 200 μL binding buffer to each tube. Finally, we analyzed the samples on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, United States).

Measurements of electrical resistance

We used an EVOM TEER meter (Millipore, Bedford, MA, United States) to monitor the transepithelial electrical resistance (TEER) of Caco-2 cells. Specifically, an increase in TEER to a steady state exceeding 200 Ω cm^2 at day 7 indicated the complete formation of tight junctions and full epithelial barrier integrity. In our experiments, we treated cell monolayers with recombinant human TNF-α (100 ng/mL) for 24 h and subsequently added 150 μg/ml TPSJ or not to the wells. Monolayers treated with cytokine alone or DMEM alone were used as controls.

Permeability study by colorimetric assay

Caco-2 cells were grown on inserts. Firstly, we washed the cell monolayers with PBS. Next, we added phenolsulfonphthalein to the apical compartment to a final concentration of 20 mg/L in ultrapure water. We added only water to the basolateral compartment. After a 4-h incubation, we removed 150 μL aliquots from the basolateral compartment into tubes containing 1.5 mL NaOH (20 μmol/mL). We then analyzed the absorbance of each tube at 570 nm using a spectrophotometer.

ELISA

We collected culture medium of from Caco-2 cells and used ELISA kits (eBioscience) to measure the amounts of TNF-α, IL-6, and IL-8 according to the manufacturer's instruction.

Immunofluorescence

We seeded Caco-2 cells on glass cover slips placed in the wells of a 6-well plate and treated the cells with TNF-α (100 ng/mL) for 24 h without or with 150 μg/mL TPSJ. The immunofluorescence assay was performed according to the protocol with antibodies specific for claudin 1, claudin 2, zo3, and occludin, followed by incubation with a FITC-conjugated anti-rabbit IgG (1:200 dilution). Images were captured using a fluorescence microscope (Olympus, BX51, Tokyo, Japan).

Measurement of NF-κB p65 activity

We used a NF-κB p50/p65 transcription factor assay kit to measure the NF-κB p65 activity in prepared cellular extracts according to the manufacturer's instructions.

Western blot

We plated Caco-2 cells in 6-well plates at a density of 1×10^6 per well. The cells were treated with recombinant human TNF-α (100 ng/mL) for 24 h without or with 150 μg/mL TPSJ for different time intervals.

At different time points, we lysed the cells in lysis buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 0.1% sodium dodecylsulfate (SDS), 1% sodium deoxycholate, 1 mmol/L phenylmethylsulphonyl fluoride, 1% Triton X-100 and protease inhibitors] and subsequently removed cell debris by centrifugation (15000 rpm, 15 min, 4 ℃). After determining the protein concentrations of the samples, we separated equal amounts of protein by 12.5% SDS-PAGE and transferred the proteins to nitrocellulose membranes. After blocking the membranes, we incubated them overnight at 4 ℃ with different primary antibodies, followed by a 1 h incubation with secondary antibodies. Finally, protein expression was evaluated using a Bio-Rad Imaging System (Bio-Rad Biosciences, Hercules, CA, United States).

Statistical analysis

The results are expressed as means ± standard errors of the means (SEM). Student's t-test was used for the statistical analysis, and a *P* value < 0.05 was considered significant. At least three independent experiments were performed.

RESULTS

TPSJ boosts the proliferation of TNF-a-damaged Caco-2 cells

We first used a MTT assay to assess the effect of TPSJ on TNF-α-damaged Caco-2 cells. As shown in Figure 2A, TPSJ dramatically induced the growth of TNF-α-treated Caco-2 cells in a dose-dependent manner, particularly at a concentration of 150 μg/mL. However, TPSJ treatment had no significant effect on the frequency of cell apoptosis in comparison to the TNF-α control group (Figure 2B and C).

TPSJ ameliorates the intestinal epithelial barrier dysfunction induced by TNF-α

Many investigators have shown that TNF-α disrupts intestinal barrier function by decreasing the TEER and increasing paracellular permeability[19,27]. Therefore, to investigate the effects of TPSJ on intestinal barrier function, we treated a Caco-2 cell monolayer with TNF-α for 24 h and subsequently analyzed the TEER and permeability of the cells after TPSJ treatment.

As shown in Figure 3A, the TEER of the TNF-α-damaged Caco-2 cell monolayer decreased significantly compared with the control group, indicating that TNF-α upregulated the paracellular permeability of ionic solutes. By contrast, TPSJ treatment significantly increased the TEER.

As shown in Figure 3B, the phenolsulfonphthalein flux was significantly higher in the TNF-α-damaged Caco-2 cell monolayer than in the control monolayer, indicating this inflammatory cytokine increased the paracellular permeability of nonionic macromolecules. However, TPSJ markedly decreased the increased phenolsulfonphthalein flux induced by TNF-α. These results suggest that TPSJ can attenuate the intestinal epithelial barrier dysfunction induced by TNF-α.

TPSJ decreased the secretion of pro-inflammatory cytokines by TNF-α-induced Caco-2 cells

The ELISA results shown in Figure 4 demonstrate significant increases in the levels of TNF-α, IL-6, and IL-8 secreted by Caco-2 cells into the culture medium after TNF-α treatment. However, TPSJ markedly decreased the secretion of these cytokines in response to TNF-α. These results indicate that TPSJ can regulate TNF-α-induced production of pro-inflammatory factors.

TPSJ protected a Caco-2 cell monolayer from TNF-α-induced barrier dysfunction by regulating tight junctions

Increasing evidence suggests that altered tight junction protein expression contributes to the proinflammatory cytokine-induced disruption of barrier function[15,28]. Therefore, we examined the effects of TPSJ on the expression of the tight junction proteins claudin 1, claudin 2, zo3, and occludin in Caco-2 cell monolayers treated with or without TNF-α. As shown in Figure 5, the expression of claudin 1, claudin 2, and zo3 proteins were significantly downregulated by TNF-α. After TPSJ treatment, however, the expressions of all three proteins were upregulated markedly at different time points. By contrast, the expression of occludin was not significantly affected by treatment with or without TNF-α or in the absence or presence of TPSJ.

Reports have demonstrated an association of proinflammatory cytokine-induced intestinal barrier dysfunction with the morphological alterations and relocalization of the tight junction[29-31]. Thus, we next determined whether TPSJ affected the morphological localization of tight junctions in Caco-2 cell monolayers treated with or without TNF-α. As shown in Figure 6, claudin 1, claudin 2, zo3, and occludin were localized along the edges of cells in the control group. However, a 24-h treatment with TNF-α rendered the tight junction distribution irregular and discontinuous, and led to the partial internalization of occludin into cytoplasmic vesicles. After TPSJ treatment,
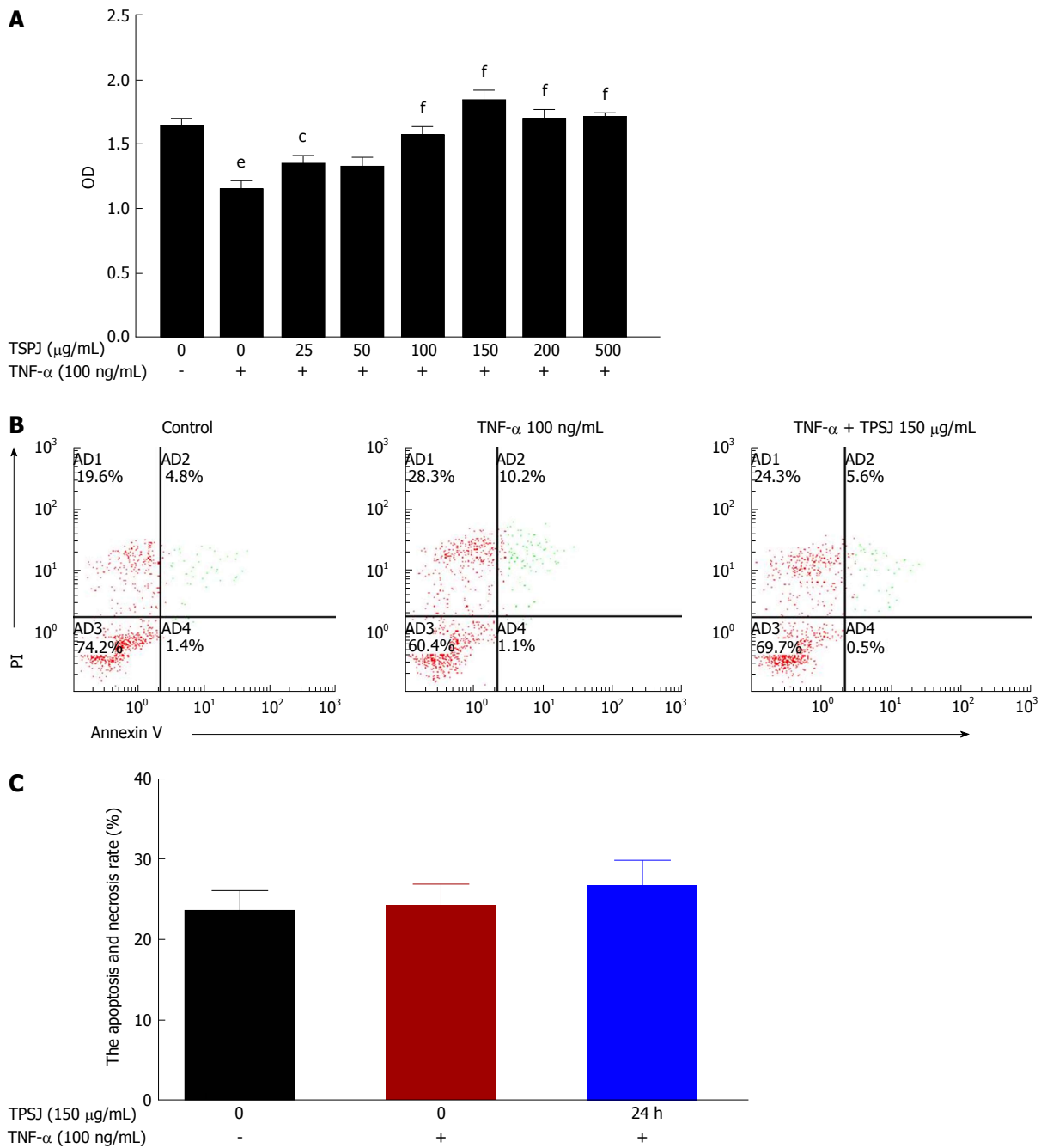
Figure 2 Total polysaccharides of the Sijunzi decoction promoted the proliferation of tumor necrosis factor-α-damaged Caco-2 cells. A: Caco-2 cells were treated with various concentrations of TPSJ (25-500 μg/mL) or control DMEM for 24 h. The effect of TPSJ on cell viability was measured using the MTT assay; B: TNF-α-damaged Caco-2 cells were incubated with 150 μg/mL TPSJ or control DMEM for 24 h. The induction of apoptosis was determined using an Annexin V-FITC/propidium iodide staining assay; C: Quantification of the numbers of apoptotic and necrotic cells. Data are shown as the means $\pm$ standard errors of the means of at least three independent experiments ([e]$P < 0.001$ *vs* control Caco-2 cells; [c]$P < 0.05$, [f]$P < 0.001$ *vs* TNF-α-damaged Caco-2 cells). TPSJ: Total polysacc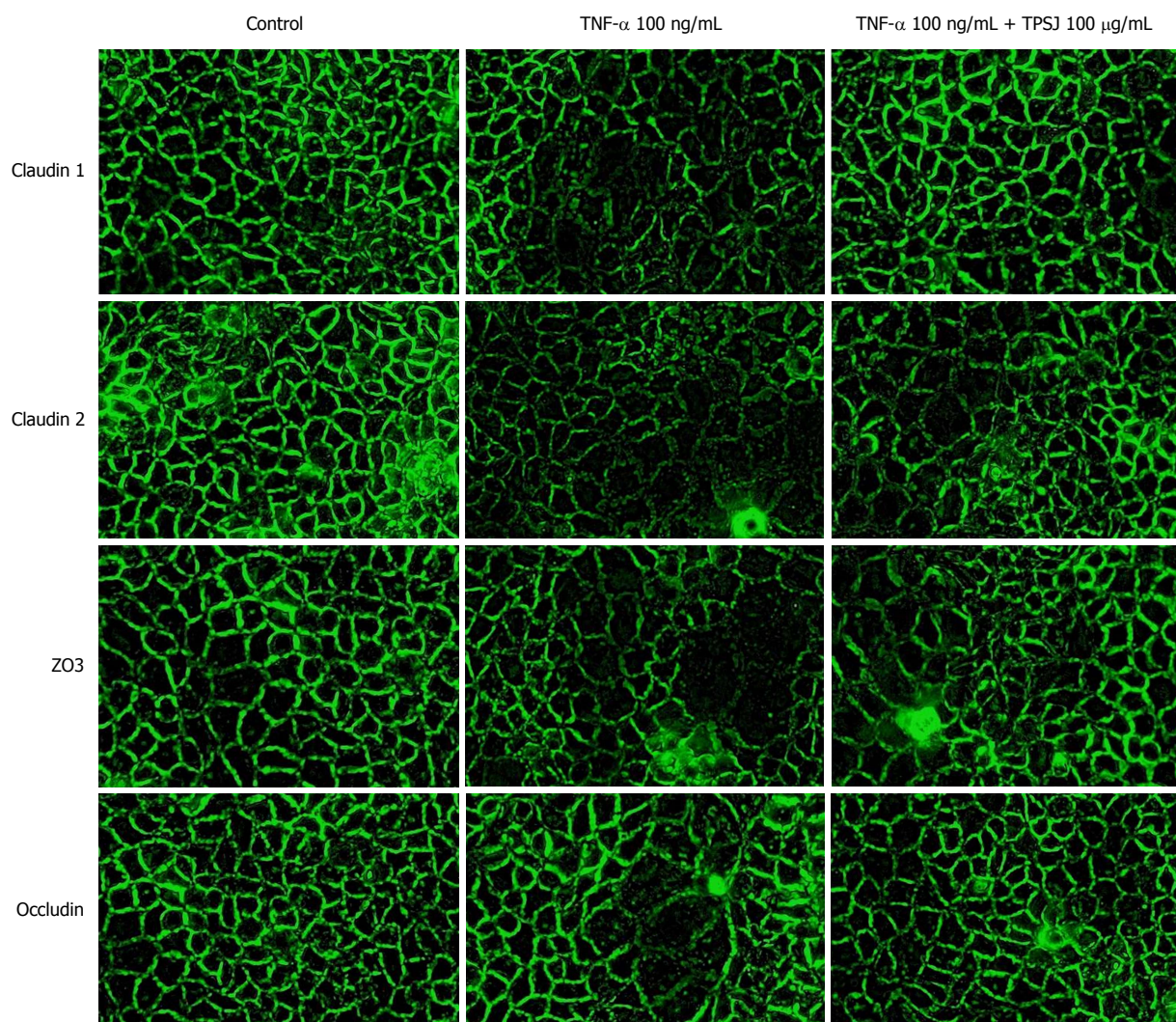harides of the Sijunzi decoction; TNF: Tumor necrosis factor; DMEM: Dulbecco's modified Eagle's medium; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

however, the reorganization of claudin 1, claudin 2, zo3, and occludin was significantly attenuated. These results indicate that TPSJ can prevent the proinflammatory cytokine-induced reorganization of tight junctions in a Caco-2 cell monolayer.

TPSJ suppresses TNF-α-induced upregulation of MLC phosphorylation and MLCK expression

The MLCK-mediated phosphorylation of MLC has been reported to play a crucial role in the regulation of intes-tinal epithelial tight junctions and paracellular leakage pathways[3,4]. Given the protective effect of TPSJ on intestinal barrier function, we wished to explore whether TPSJ could alleviate TNF-α-induced barrier dysfunction and tight junction disruption by inhibiting MLC phospho-rylation. As shown in Figure 7A and B, treatment of a Caco-2 monolayer with TNF-α induced a significant increase in the ratio of phosphorylated to total MLC. However, TPSJ treatment markedly downregulated this ratio at 6 h and 9 h (compared with the TNF-α control

Figure 3 Total polysaccharides of the Sijunzi decoction attenuated intestinal epithelial barrier dysfunction induced by tumor necrosis factor-α. A: Caco-2 monolayers were incubated with or without 100 ng/mL TNF-α in the absence or presence of TPSJ for 24 h. TPSJ significantly increased the transepithelial electrical resistance of Caco-2 cell monolayers damaged by TNF-α; B: TPSJ markedly attenuated phenolsulfonphthalein flux across the epithelial monolayers. Data are shown as the means ± standard errors of the means of at least three independent experiments ([b]$P < 0.01$ *vs* control Caco-2 cells; [c]$P < 0.05$ *vs* TNF-α-damaged Caco-2 cells). TPSJ: Total polysaccharides of the Sijunzi decoction; TNF: Tumor necrosis factor.

Figure 4 Total polysaccharides of the Sijunzi decoction regulated the secretion of inflammatory cytokines by Caco-2 cells. Caco-2 cells were incubated with or without 100 ng/mL TNF-α in the absence or presence of TPSJ for 24 h. Cell culture media were collected, and the concentrations of TNF-α (A), IL-6 (B), and IL-8 (C) were detected by ELISA ([b]$P < 0.01$, [e]$P < 0.001$ *vs* control Caco-2 cells; [c]$P < 0.05$, [f]$P < 0.001$ *vs* TNF-α-damaged Caco-2 cells). TPSJ: Total polysaccharides of the Sijunzi decoction; TNF: Tumor necrosis factor.

group).

MLCK is a well-known and predominant regulator of MLC phosphorylation, and many studies have indicated an association of MLCK upregulation with tight junction dysfunction and paracellular hyperpermeability[30,31]. Therefore, we next investigated the effect of TPSJ on MLCK expression in Caco-2 cell monolayers treated with or without TNF-α. As shown in Figure 7C and D, MLCK expression increased significantly in the TNF-α-treated monolayer. However, TPSJ treatment significantly reduced MLCK expression at 9 h, compared with the

TNF-α control group. These results suggest that TPSJ attenuates TNF-α-induced intestinal barrier disruption by suppressing the MLCK-mediated phosphorylation of MLC.

TPSJ attenuated TNF-α-induced intestinal epithelial barrier dysfunction by inhibiting NF-κB p65

Studies have shown that NF-κB activation plays a role in intestinal barrier dysfunction as well as in the upregulation of MLCK in TNF-α treated intestinal epithelial cells[32,33]. Based on the above results, we aimed to investigate further the potential involvement of the NF-κB signa-

Figure 5 Total polysaccharides of the Sijunzi decoction modified the expression of claudin 1, claudin 2, zo3, and occludin. A: A representative western blot of claudin 1 expression in TNF-α-damaged Caco2 cells treated with TPSJ for 12 h, 24 h, and 48 h; B: Quantification of the amounts of claudin 2 relative to GAPDH at different time points; C: A representative western blot of claudin 2 in TNF-α-damaged Caco-2 cells treated with TPSJ for 12 h, 24 h, and 48 h; D: Quantification of the amounts of claudin 2 relative to GAPDH at different time points; E: A representative western blot of zo3 in TNF-α-damaged Caco-2 cells treated with TPSJ for 12 h, 24 h, and 48 h; F: Quantification of the amounts of zo3 relative to GAPDH at different time points; G: A representative western blot of occludin in TNF-α-damaged Caco-2 cells treated with TPSJ for 12 h, 24 h, and 48 h. H: Quantification of the amounts of occludin relative to GAPDH at different time points. Data are shown as the means ± standard errors of the means of at least three independent experiments ([a]$P < 0.05$, [b]$P < 0.01$ *vs* control Caco-2 cells; [c]$P < 0.05$, [d]$P < 0.01$ *vs* TNF-α-damaged Caco-2 cells). TPSJ: Total polysaccharides of the Sijunzi decoction; TNF: Tumor necrosis factor.

Figure 6 Immunofluorescence analysis of the effects of total polysaccharides of the Sijunzi decoction on the tight junction proteins claudin 1, claudin 2, zo3, and occludin in tumor necrosis factor-α-damaged Caco-2 cells. Results are representative of three independent experiments. Original magnification = 400 ×.

ling pathway in the protective effect of TPSJ against TNF-α-induced epithelial barrier dysfunction. As demonstrated in Figure 8A, NF-κB p65 activity was upregu-lated in the TNF-α-treated Caco-2 cell monolayer. TPSJ treatment, however, significantly inhibited NF-κB p65 activity at 6 h and 9 h compared with the TNF-α-treated Caco-2 mono-layer. Similarly, as shown in Figure 8B and C, the nuclear expression of NF-κB p65 increased following TNF-α treatment, whereas TPSJ markedly downregulated the nuclear expression at 6 h and 9 h.

DISCUSSION

Inflammatory bowel diseases, including UC and CD, are well-known chronic and recurring inflammatory diseases of the intestinal tract. Although the pathogenesis of inflammatory bowel diseases is not fully elucidated, all are characterized by the overproduction of proinflammatory cytokines within the mucosa and the disruption of epithelial barrier function. However, many research groups have demonstrated that proinflammatory cytokines

may disrupt intestinal barrier function both *in vivo* and *in vitro*[28,29,34]. Therefore, restoration of the intestinal barrier function is a worthwhile strategy for the treatment of inflammatory bowel diseases.

As noted above, TPSJ was previously reported to antagonize cyclophosphamide-induced mucosal-associated lymphoid tissue injury in an animal model, suggesting a potential beneficial role in intestinal mucosal immune function[24]. In this study, we showed that TPSJ could attenuate intestinal epithelial barrier dysfunction caused by proinflammatory cytokines in a Caco-2 cell monolayer. Specifically, TPSJ alleviated the TNF-α-induced decrease in TEER and increase in paracellular permeability, enhanced the expression of claudin 1, claudin 2, and zo3, and preserved the morphological distributions of these three tight junction proteins and occludin.

The molecular mechanism by which TPSJ ameliorates the proinflammatory cytokine-induced intestinal barrier dysfunction is currently unknown. Many research groups have demonstrated that the upregulation of MLCK and subsequent increase in MLC phosphorylation are es-

Figure 7 Total polysaccharides of the Sijunzi decoction inhibited tumor necrosis factor-α-induced increases in myosin light chain phosphorylation and myosin light chain kinase protein expression. A: A representative western blot of pMLC and MLC in TNF-α-damaged Caco-2 cells treated with TPSJ; B: Quantification of the amounts of pMLC relative to MLC; C: A representative western blot of MLCK in TNF-α-damaged Caco-2 cells treated with TPSJ; D: Quantification of the amounts of MLCK relative to GAPDH. Data are shown as the means ± standard errors of the means of at least three independent experiments ([a]$P < 0.05$ *vs* control Caco-2 cells; [c]$P < 0.05$, [d]$P < 0.01$ *vs* TNF-α-damaged Caco-2 cells). TPSJ: Total polysaccharides of the Sijunzi decoction; MLC: Myosin light chain; MLCK: MLC kinase; TNF: Tumor necrosis factor.

Figure 8 Effects of total polysaccharides of the Sijunzi decoction on the nuclear factor-κB signaling pathway. A: TPSJ upregulated the activity of the NF-κB transcription factor p65; B: A representative western blot of NF-κB p65 in TNF-α-damaged Caco-2 cells treated with TPSJ; C: Quantification of the amounts of p65 relative to PCNA. Data are shown as the means ± standard errors of at least three independent experiments [b]$P < 0.01$ *vs* control Caco-2 cells; [c]$P < 0.05$, [d]$P < 0.01$ *vs* TNF-α-damaged Caco-2 cells). TPSJ: Total polysaccharides of the Sijunzi decoction; NF: Nuclear factor; TNF: Tumor necrosis factor.

sential to the induction of intestinal barrier defects by proinflammatory cytokines[9,12]. In our study, we found that TPSJ inhibited increases in MLC phosphorylation and MLCK expression in Caco-2 cell monolayers exposed to TNF-α, suggesting that TPSJ may attenuate proinflammatory cytokine-induced intestinal barrier dysfunction by inhibiting MLCK activation and subsequent MLC phosphorylation. However, other potential molecular mechanisms remain to be investigated further.

According to previously published reports, activated NF-κB mediates the increased intestinal epithelial tight junction permeability induced by TNF-α[27] and contributes to MLCK upregulation in a Caco-2 cell monolayer exposed to proinflammatory cytokines[12,32]. In this study, we showed that TPSJ could suppress the activation and expression of NF-κB p65 in a Caco-2 cell monolayer treated with TNF-α. Our results suggest that the mechanism by which TPSJ attenuates TNF-α-induced barrier dysfunction in the Caco-2 cell monolayer is mediated by the NF-κB signaling pathway.

In conclusion, our results demonstrate that TPSJ attenuates the intestinal barrier dysfunction elicited by TNF-α treatment in a Caco-2 cell monolayer. We further demonstrate that TPSJ inhibits the TNF-α-induced upregulation of MLC phosphorylation, which is mediated by MLCK and NF-κB. These factors may comprise the mechanism by which TPSJ protects the intestinal epithelial barrier from destruction triggered by proinflammatory cytokines.

ARTICLE HIGHLIGHTS

Research background
Sijunzi decoction (SJZD) is a traditional Chinese medicinal prescription that has been used to treat gastrointestinal tract diseases since ancient times. Our previous studies suggested that total polysaccharides of the Sijunzi decoction (TPSJ) could inhibit the proliferation of IEC-6 rat intestinal epithelial cells *in vitro*. However, no report has discussed the regulatory effects of TPSJ on the intestinal epithelial barrier.

Research motivation
Although TPSJ may inhibit intestinal epithelial cell proliferation, the mechanism by which it mediates barrier protection remains unclear.

Research objectives
To explore the protective effects of TPSJ on the epithelial barrier and the mechanism by which it mitigates tumor necrosis factor α (TNF-α)-induced damage in a Caco-2 cell monolayer.

Research methods
We first used a MTT assay to assess the effect of TPSJ on TNF-α-damaged Caco-2 cells. Secondly, we treated a Caco-2 cell monolayer with TNF-α for 24 h and subsequently analyzed the TEER, permeability, and cytokines of the cells after TPSJ treatment. Third, we examined the effects of TPSJ on the expression of the tight junction proteins claudin 1, claudin 2, zo3, and occludin by immunofluorescence and western blotting. Finally, we investigated the NF-κB-MLCK-MLC pathway in TNF-α treated intestinal epithelial cells.

Research results
TPSJ promoted the growth of TNF-α-treated Caco-2 cells in a dose-dependent manner and decreased the secretion of pro-inflammatory cytokines in response to TNF-α. Secondly, TPSJ treatment significantly increased the TEER and

decreased the increased phenolsulfonphthalein flux induced by TNF-α. Third, TPSJ markedly upregulated the expression of claudin 1, claudin 2, and zo3 proteins and attenuated the reorganization of claudin 1, claudin 2, zo3, and occludin. Finally, TPSJ suppressed the TNF-a-induced upregulation of myosin light chain (MLC) phosphorylation, MLC kinase (MLCK), and NF-κB p65.

Research conclusions
TPSJ promoted proliferation of TNF-α-treated Caco2 cells. In Caco2 cell monolayers, TPSJ alleviated the TNF-α-induced decrease in TEER and increase in paracellular permeability, enhanced the expression of claudin 1, claudin 2, and zo3, and preserved the morphological distributions of these three tight junction proteins and occludin. Further, we found that the barrier protective effect of TPSJ was mediated through suppressing the NF-κB p65-mediated phosphorylation of MLCK and MLC.

Research perspectives
Our findings provide evidence that TPSJ is a potential protective agent of intestinal barrier function. Further investigation into the mechanism of TPSJ on intestinal barrier as well as *in vivo* research is required.

REFERENCES

1 **Arnott ID**, Kingstone K, Ghosh S. Abnormal intestinal permeability predicts relapse in inactive Crohn disease. *Scand J Gastroenterol* 2000; **35**: 1163-1169 [PMID: 11145287 DOI: 10.1080/003655200750056637]

2 **Wyatt J**, Vogelsang H, Hübl W, Waldhöer T, Lochs H. Intestinal permeability and the prediction of relapse in Crohn's disease. *Lancet* 1993; **341**: 1437-1439 [PMID: 8099141 DOI: 10.1016/0140-6736(93)90882-H]

3 **Shen L**, Weber CR, Raleigh DR, Yu D, Turner JR. Tight junction pore and leak pathways: a dynamic duo. *Annu Rev Physiol* 2011; **73**: 283-309 [PMID: 20936941 DOI: 10.1146/annurev-physiol-012110-142150]

4 **Turner JR**. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol* 2009; **9**: 799-809 [PMID: 19855405 DOI: 10.1038/nri2653]

5 **Weber CR**. Dynamic properties of the tight junction barrier. *Ann N Y Acad Sci* 2012; **1257**: 77-84 [PMID: 22671592 DOI: 10.1111/j.1749-6632.2012.06528.x]

6 **Rutgeerts P**, Sandborn WJ, Feagan BG, Reinisch W, Olson A, Johanns J, Travers S, Rachmilewitz D, Hanauer SB, Lichtenstein GR, de Villiers WJ, Present D, Sands BE, Colombel JF. Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med* 2005; **353**: 2462-2476 [PMID: 16339095 DOI: 10.1056/NEJMoa050516]

7 **Targan SR**, Hanauer SB, van Deventer SJ, Mayer L, Present DH, Braakman T, DeWoody KL, Schaible TF, Rutgeerts PJ. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N Engl J Med* 1997; **337**: 1029-1035 [PMID: 9321530 DOI: 10.1056/NEJM199710093371502]

8 **Martínez C**, González-Castro A, Vicario M, Santos J. Cellular and molecular basis of intestinal barrier dysfunction in the irritable bowel syndrome. *Gut Liver* 2012; **6**: 305-315 [PMID: 22844557 DOI: 10.5009/gnl.2012.6.3.305]

9 **McGuckin MA**, Eri R, Simms LA, Florin TH, Radford-Smith G. Intestinal barrier dysfunction in inflammatory bowel diseases. *Inflamm Bowel Dis* 2009; **15**: 100-113 [PMID: 18623167 DOI: 10.1002/ibd.20539]

10 **Salim SY**, Söderholm JD. Importance of disrupted intestinal barrier in inflammatory bowel diseases. *Inflamm Bowel Dis* 2011; **17**: 362-381 [PMID: 20725949 DOI: 10.1002/ibd.21403]

11 **Suzuki T**. Regulation of intestinal epithelial permeability by tight junctions. *Cell Mol Life Sci* 2013; **70**: 631-659 [PMID: 22782113 DOI: 10.1007/s00018-012-1070-x]

12 **Rafa H**, Benkhelifa S, AitYounes S, Saoula H, Belhadef S, Belkhelfa M, Boukercha A, Toumi R, Soufli I, Moralès O, de Launoit Y, Mahfouf H, Nakmouche M, Delhem N, Touil-Boukoffa C. All-

Trans Retinoic Acid Modulates TLR4/NF-κB Signaling Pathway Targeting TNF-α and Nitric Oxide Synthase 2 Expression in Colonic Mucosa during Ulcerative Colitis and Colitis Associated Cancer. *Mediators Inflamm* 2017; **2017**: 7353252 [PMID: 28408791 DOI: 10.1155/2017/7353252]

13   **Toumi R**, Soufli I, Rafa H, Belkhelfa M, Biad A, Touil-Boukoffa C. Probiotic bacteria lactobacillus and bifidobacterium attenuate inflammation in dextran sulfate sodium-induced experimental colitis in mice. *Int J Immunopathol Pharmacol* 2014; **27**: 615-627 [PMID: 25572742 DOI: 10.1177/039463201402700418]

14   **Soufli I**, Toumi R, Rafa H, Touil-Boukoffa C. Overview of cytokines and nitric oxide involvement in immuno-pathogenesis of inflammatory bowel diseases. *World J Gastrointest Pharmacol Ther* 2016; **7**: 353-360 [PMID: 27602236 DOI: 10.4292/wjgpt.v7.i3.353]

15   **Suzuki T**, Yoshinaga N, Tanabe S. Interleukin-6 (IL-6) regulates claudin-2 expression and tight junction permeability in intestinal epithelium. *J Biol Chem* 2011; **286**: 31263-31271 [PMID: 21771795 DOI: 10.1074/jbc.M111.238147]

16   **Wang F**, Graham WV, Wang Y, Witkowski ED, Schwarz BT, Turner JR. Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. *Am J Pathol* 2005; **166**: 409-419 [PMID: 15681825 DOI: 10.1016/S0002-9440(10)62264-X]

17   **Wang F**, Schwarz BT, Graham WV, Wang Y, Su L, Clayburgh DR, Abraham C, Turner JR. IFN-gamma-induced TNFR2 expression is required for TNF-dependent intestinal epithelial barrier dysfunction. *Gastroenterology* 2006; **131**: 1153-1163 [PMID: 17030185 DOI: 10.1053/j.gastro.2006.08.022]

18   **Weber CR**, Raleigh DR, Su L, Shen L, Sullivan EA, Wang Y, Turner JR. Epithelial myosin light chain kinase activation induces mucosal interleukin-13 expression to alter tight junction ion selectivity. *J Biol Chem* 2010; **285**: 12037-12046 [PMID: 20177070 DOI: 10.1074/jbc.M109.064808]

19   **Schwarz BT**, Wang F, Shen L, Clayburgh DR, Su L, Wang Y, Fu YX, Turner JR. LIGHT signals directly to intestinal epithelia to cause barrier dysfunction via cytoskeletal and endocytic mechanisms. *Gastroenterology* 2007; **132**: 2383-2394 [PMID: 17570213 DOI: 10.1053/j.gastro.2007.02.052]

20   **Cao J**. 49 cases of Sijunzi decoction on functional dyspepsia of spleen deficiency and liver stagnation syndrome *Zhongguo Zhongxiyi Jiehe Xiaohua Zazhi* 2008; **16**: 126-128

21   **Chen J**. Effect of Sijunzi decoction and enteral nutrition in postoperative recovery of gastric cancer. *Jilin Zhongyiyao* 2013; **33**: 383-385

22   **Li Y**. Clinical observation of Sijunzi decoction in the treatment of spleen and stomach types of functional dyspepsia. *Beijing Zhongyiyao* 2008; **27**: 806-807

23   **Shan T**, Yu XY, Zhou ZL, Xie JL, Li L. Effect of Sijunzi decoction on restoration of gut barrier after relief of intestinal obstruction in rabbit intestine. *Zhongguo Zhongxiyi Jiehe Waike Zazhi* 2010; **16**: 319-323

24   **Liu L**, Zhou H, Wang PX, Hu YJ. Influence of total polysaccharide extracted from Sijunzi decoction on the intestinal mucosa-associated lymphoid tissues of mice. *Zhongguo Mianyixue Zazhi* 2000; **17**: 204-206

25   **Liu L**, Han L, Wong DY, Yue PY, Ha WY, Hu YH, Wang PX, Wong RN. Effects of Si-Jun-Zi decoction polysaccharides on cell migration and gene expression in wounded rat intestinal epithelial cells. *Br J Nutr* 2005; **93**: 21-29 [PMID: 15705221 DOI: 10.1079/BJN20041295]

26   **Deng J**, Li RL, Cai JZ, Tu XH, Chen WW. Changes in IEC-6 cell migration ability are impacted by polysaccharides separated and purified from Sijunzi decoction. *World Sci Tech/ Modern Trad Chin Med Mat Medi* 2016; **18**: 600-606

27   **Ma TY**, Iwamoto GK, Hoa NT, Akotia V, Pedram A, Boivin MA, Said HM. TNF-alpha-induced increase in intestinal epithelial tight junction permeability requires NF-kappa B activation. *Am J Physiol Gastrointest Liver Physiol* 2004; **286**: G367-G376 [PMID: 14766535 DOI: 10.1152/ajpgi.00173.2003]

28   **Amasheh M**, Grotjohann I, Amasheh S, Fromm A, Söderholm JD, Zeitz M, Fromm M, Schulzke JD. Regulation of mucosal structure and barrier function in rat colon exposed to tumor necrosis factor alpha and interferon gamma in vitro: a novel model for studying the pathomechanisms of inflammatory bowel disease cytokines. *Scand J Gastroenterol* 2009; **44**: 1226-1235 [PMID: 19658020 DOI: 10.1080/00365520903131973]

29   **Li Q**, Zhang Q, Wang M, Zhao S, Ma J, Luo N, Li N, Li Y, Xu G, Li J. Interferon-gamma and tumor necrosis factor-alpha disrupt epithelial barrier function by altering lipid composition in membrane microdomains of tight junction. *Clin Immunol* 2008; **126**: 67-80 [PMID: 17964857 DOI: 10.1016/j.clim.2007.08.017]

30   **Liu H**, Li M, Wang P, Wang F. Blockade of hypoxia-inducible factor-1α by YC-1 attenuates interferon-γ and tumor necrosis factor-α-induced intestinal epithelial barrier dysfunction. *Cytokine* 2011; **56**: 581-588 [PMID: 21890376 DOI: 10.1016/j.cyto.2011.08.023]

31   **Liu H**, Wang P, Cao M, Li M, Wang F. Protective role of oligomycin against intestinal epithelial barrier dysfunction caused by IFN-γ and TNF-α. *Cell Physiol Biochem* 2012; **29**: 799-808 [PMID: 22613980 DOI: 10.1159/000188076]

32   **Graham WV**, Wang F, Clayburgh DR, Cheng JX, Yoon B, Wang Y, Lin A, Turner JR. Tumor necrosis factor-induced long myosin light chain kinase transcription is regulated by differentiation-dependent signaling events. Characterization of the human long myosin light chain kinase promoter. *J Biol Chem* 2006; **281**: 26205-26215 [PMID: 16835238 DOI: 10.1074/jbc.M602164200]

33   **Ye D**, Ma TY. Cellular and molecular mechanisms that mediate basal and tumour necrosis factor-alpha-induced regulation of myosin light chain kinase gene activity. *J Cell Mol Med* 2008; **12**: 1331-1346 [PMID: 18363837 DOI: 10.1111/j.1582-4934.2008.00302.x]

34   **Ma TY**, Boivin MA, Ye D, Pedram A, Said HM. Mechanism of TNF-{alpha} modulation of Caco-2 intestinal epithelial tight junction barrier: role of myosin light-chain kinase protein expression. *Am J Physiol Gastrointest Liver Physiol* 2005; **288**: G422-G430 [PMID: 15701621 DOI: 10.1152/ajpgi.00412.2004]

Retrospective Study

Efficacy and safety of endoscopic submucosal tunnel dissection for superficial esophageal squamous cell carcinoma and precancerous lesions

Jin Wang, Xiao-Nan Zhu, Lin-Lin Zhu, Wei Chen, Yi-Han Ma, Tao Gan, Jin-Lin Yang

Jin Wang, Xiao-Nan Zhu, Wei Chen, Yi-Han Ma, Tao Gan, Jin-Lin Yang, Department of Gastroenterology and Hepatology, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China

Jin Wang, State Key Laboratory of Biotherapy and Cancer Center, Sichuan University, Chengdu 610041, Sichuan Province, China

Lin-Lin Zhu, Department of General Practice, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China

ORCID number: Jin Wang (0000-0003-0944-0793); Xiao-Nan Zhu (0000-0001-5979-2143); Lin-Lin Zhu (0000-0002-4740-2838); Wei Chen (0000-0003-1905-8408); Yi-Han Ma (0000-0003-4605-0217); Tao Gan (0000-0002-7331-062X); Jin-Lin Yang (0000-0001-8726-7258).

Author contributions: Wang J, Zhu LL, Gan T and Yang JL designed the research; Wang J, Zhu XN, Zhu LL, Chen W and Ma YH performed the research; Wang J, Zhu XN, Chen W and Ma YH collected the data; Wang J, Zhu LL and Gan T analyzed the data; Wang J, Zhu LL and Yang JL wrote the paper.

Correspondence to: Jin-Lin Yang, MD, PhD, Chief Doctor, Doctor, MHSc, Professor, Department of Gastroenterology and Hepatology, West China Hospital of Sichuan University, 37th Guoxue Alley, Chengdu 610041, Sichuan Province, China. mouse-577@163.com
Telephone: +86-28-85423326
Fax: +86-28-85423326

Abstract

AIM
To evaluate the clinical outcomes of patients who underwent endoscopic submucosal tunnel dissection (ESTD) for esophageal squamous cell carcinoma (ESCC) and precancerous lesions.

METHODS
ESTD was performed in 289 patients. The clinical outcomes of the patients and pathological features of the lesions were retrospectively reviewed.

RESULTS

A total of 311 lesions were included in the analysis. The en bloc rate, complete resection rate, and curative resection rate were 99.04%, 81.28%, and 78.46%, respectively. The ESTD procedure time was 102.4 ± 35.1 min, the mean hospitalization time was 10.3 ± 2.8 d, and the average expenditure was 3766.5 ± 846.5 dollars. The intraoperative bleeding rate was 6.43%, the postoperative bleeding rate was 1.61%, the perforation rate was 1.93%, and the postoperative infection rate was 9.65%. Esophageal stricture and positive margin were severe adverse events, with an incidence rate of 14.79% and 15.76%, respectively. No tumor recurrence occurred during the follow-up period.

CONCLUSION

ESTD for ESCC and precancerous lesions is feasible and relatively safe, but for large mucosal lesions, the rate of esophageal stricture and positive margin is high.

Key words: Superficial esophageal squamous cell carcinoma; Endoscopic submucosal tunnel dissection; Efficiency; Safety; Esophageal stricture

Core tip: Endoscopic submucosal tunnel dissection (ESTD) is a modified technique based on endoscopic submucosal dissection. In this paper, we found ESTD is feasible and relatively safe for treating esophageal squamous cell carcinoma (ESCC) and precancerous lesions. The en bloc rate was high, while the adverse event rate was relatively low. When treating large mucosal lesions, ESTD has a high rate of esophageal stricture and positive margin, which requires further treatment. Furthermore, we found that the pathology of preoperative biopsies had to be upgraded after ESTD, which suggests that the accuracy of biopsy to diagnose ESCC should be reconsidered.

INTRODUCTION

Endoscopic submucosal dissection (ESD) is becoming the standard treatment for early gastrointestinal cancers, as it has a higher en bloc resection rate and a lower recurrence rate than endoscopic mucosal resection (EMR)[1,2] and it can be used to resect lesions with a diameter greater than 2 cm[3]. However, esophageal ESD faces many difficulties because of the narrow esophageal lumen and thin walls[4-6]. When using conventional ESD treatment for large mucosal lesions, and especially for lesions with a circumference that exceeds three fourths of the esophageal lumen, multiple submucosal injections are required, which could prolong the procedure time and thereby increase the risk of complications[5]. Even worse, with the resected mucosa blocked in the lumen, the endoscopic view becomes unclear and may increase the difficulty of complete resection[6]. To overcome these difficulties, some modified ESD techniques have been introduced, such as the line traction method[6], the clip traction method[7], and the thread-traction method[8]; however, none of these methods was suitable for extensive application.

In 2009, Linghu *et al*[9] used a "submucosal tunnel" to resect successfully a circumferential esophageal mucosal lesion, which was subsequently termed an endoscopic submucosal tunnel dissection (ESTD)[5]. Compared with conventional ESD, ESTD has many technical advantages, as it resects the mucosal lesions by creating a submucosal tunnel between the mucosal layer and muscular layer, after which therapeutic endoscopy can enter the tunnel and acquire a clear operative view. Moreover, the CO_2 injected in the operation can help the blunt dissection of the mucosal layer, thereby reducing the number of submucosal injections, shortening the procedure time, increasing the resection speed, and reducing the injury of the muscular layer[10-12]. This approach can also incise the submucosa more completely, thereby reducing the risk of tumor metastasis and recurrence, as shown in our previous study[13]. However, there are no studies that have verified the feasibility of ESTD in superficial esophageal squamous cell carcinoma (ESCC) and precancerous lesions in a large sample. The aim of this study was to assess the efficacy and safety of ESTD in treating superficial ESCC and precancerous lesions in a relatively large sample.

MATERIALS AND METHODS

Patients and endoscopic characteristics

A prospectively collected endoscopic therapy database was analyzed retrospectively. All of the patients with superficial ESCC and precancerous lesions who underwent ESTD in the Digestive Endoscopy Center of West China Hospital from March 1, 2013 to May 1, 2017 were enrolled. A total of 355 patients with superficial esophageal cancer underwent endoscopic treatment. We excluded patients with esophageal adenocarcinoma, EMR/ESD procedure, and incomplete clinical data. Finally, 289 patients with 311 lesions were analyzed (Figure 1). All of the lesions were confirmed by pathological evaluation from biopsy specimens according to the Japanese classification of ESCC[14] before ESTD procedure. The clinical records and ESTD records were collected, and demographic and endoscopic characteristics were retrospectively reviewed. The endoscopic type of lesion was assessed according to the Paris endoscopic classification[15]. This study was approved
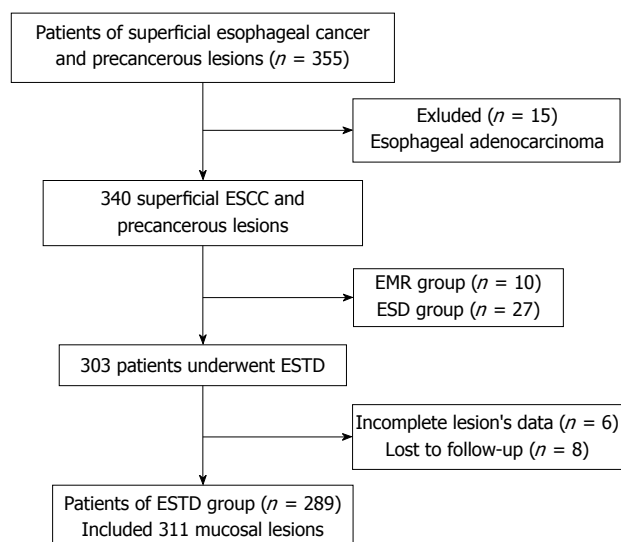
Figure 1 Flowchart of the enrollment process. ESCC: Esophageal squamous cell carcinoma; ESTD: Endoscopic submucosal tunnel dissection; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection

by the Ethics Committee of the West China Hospital of Sichuan University.

ESTD procedure

Before ESTD, all of the lesions were evaluated by endoscopy, enhanced ultrasound (EUS), and computed tomography (CT) of chest and abdomen. Prophylactic antibiotics were used in patients with large mucosal lesions (circumference ⩾ 3/4) at half an hour before ESTD. ESTDs were performed by one endoscopist with an experience of more than 200 cases of ESD procedure. ESTD procedure included six steps, as shown in Figure 2. When the lesion was detected by white light endoscopy, it was carefully observed under narrow band imaging (NBI) and iodine staining. Next, the margins were marked by a dual knife (KD-650Q, Olympus, Tokyo, Japan). A liquid mixture of 1:10000 adrenaline saline, sodium hyaluronate, glycerin fructose, and indigo carmine was used in submucosal injection. Both anal-side and oral-side incisions were made after submucosal injection, after which the submucosal tunnel was established from the oral side to the anal side and stopped at the anal-side incision. Thereafter, the remaining lateral margin incisions were made; thus, the lesion was completely resected. Finally, wound hemostasis was carefully performed by hemostatic forceps (FD-410LR, Olympus) or argon plasma coagulator (ERBE Corporation).

Postoperative strategies and follow-up

Patients were allowed to feed orally from the third day after ESTD, while treatment with proton pump inhibitors, hemostatics, and nutritional supports was initiated. The vital signs were monitored, and gas-related complications were closely detected, including subcutaneous emphysema, mediastinal emphysema, and pneumoperitoneum. All patients were asked to join in the follow-up plan, and surveillance endoscopy with iodine staining was performed at 1, 3, 6, 12, 24, and 36 mo after ESTD. Biopsies for suspicious lesions were also recommended. The patients with non-curative resection underwent either additional treatment (re-ESD, radiotherapy, surgery) or close surveillance.

Outcome measures

The primary outcomes included en bloc resection rate, complete resection rate, and curative resection rate as well as the data acquired from ESTD procedure, such as procedure time, dissection speed, and the specimen area. The secondary outcomes were the rates of adverse events, including intraoperative and postoperative bleeding, perforation, muscular injury, postoperative infection, esophageal stricture, positive margin, and local tumor reoccurrence. The symptom score of esophageal stricture was assessed according to Stooler's dysphagia score[16].

Definitions

Procedure time was defined as the time from lesion marking to the termination of therapeutic endoscopy. Specimen area was calculated by the formula: $S = (a + b)/2 \times (c + d)/2$, ($a$ and b represent the maximum and minimum values of the length diameter, respectively, while c and d represent the maximum and minimum values of the width diameter, respectively). En bloc resection was defined as resection of the lesion by an entire specimen, while complete resection/R0 resection was defined as an en bloc resection with neoplasia-free margins (both horizontal and vertical margins). Curative resection was pathologically defined as a complete resection with a differentiated carcinoma with < 200 μm submucosal invasion and no lympho-vascular invasion.

Intraoperative bleeding was defined as blood volume > 50 mL and bleeding that could be effectively stopped in ESTD procedure. Postoperative bleeding was defined as the symptoms of hematemesis or/and melena, with hemoglobin levels being decreased by more than 20 g/L within 30 d after ESTD procedure[17]. Perforation was defined as a visible hole in the esophageal wall or the presence of subcutaneous emphysema, pneumothorax, mediastinal emphysema, or pneumoperitoneum.

Esophageal stricture was defined when the standard GIF-Q260J (Olympus) gastroscopy could not pass through the esophageal lumen and if the patient had dysphagia[18]. A positive margin was defined as the presence of a neoplastic cell in the horizontal or vertical margins. Residual tumor was defined as the presence of new tumor lesions in the primary resection site and its surrounding 1 cm area within 6 mo after ESTD. Tumor reoccurrence was defined as the presence of new tumor lesions in the primary resection site and its surrounding 1 cm area over 6 mo after ESTD.

Statistical analysis

Continuous variables are represented by average ± SD and were compared by Student's *t*-test. Categorical variables are represented by the rate and evaluated by Pearson Chi square test or Fisher exact test (SPSS
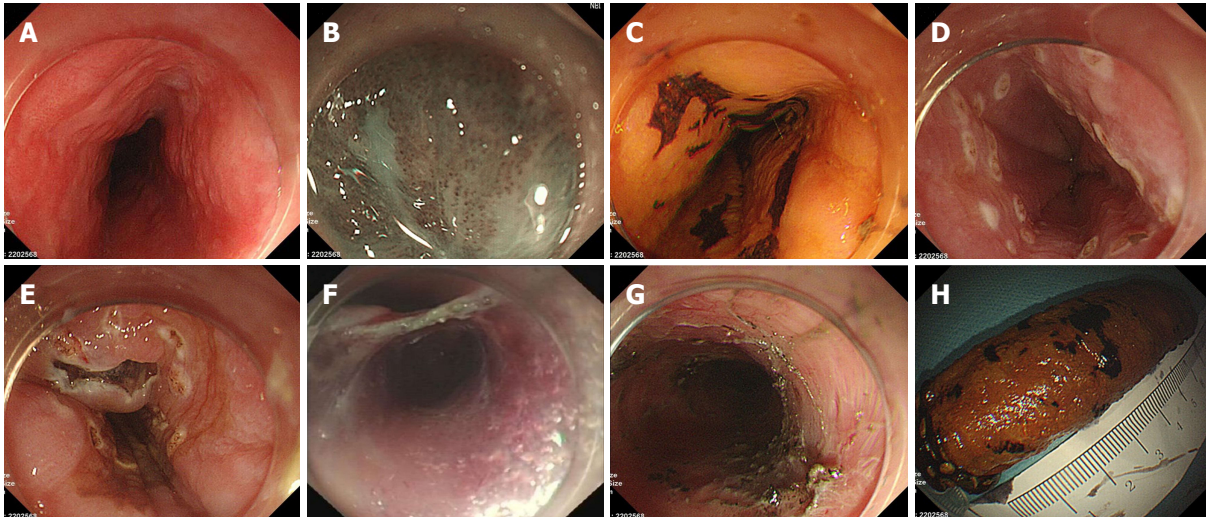
Figure 2 Endoscopic submucosal tunnel dissection procedures. A: Lesion was detected under white light endoscopy; B: Lesion was observed under narrow band imaging (NBI); C: Lesion was observed under iodine staining; D: The margin of the lesion was marked; E: Anal-side and oral-side incisions after submucosal injection; F: Creating the submucosal tunnel and resecting the lesion; G: The artificial wound after endoscopic submucosal tunnel dissection; H: The *in vitro* specimen encircled in the body of a syringe after iodine staining.

Table 1 Baseline characteristics of 311 lesions treated with endoscopic submucosal tunnel dissection n (%)

Category	ESTD (n = 311)
Sex, male/female	213/98
Age, yr, mean (range)	61.4 ± 8.1 (40-83)
Tumor location	
Upper third	24 (7.72)
Middle third	200 (64.31)
Lower third	87 (27.97)
Paris classification	
0- I	18 (5.79)
0- II a	111 (35.69)
0- II b	94 (30.23)
0- II c	35 (11.25)
0- II a- II c	50 (16.08)
0- III	3 (0.96)
Circumferential level	
≤ 1/4	11 (3.54)
≤ 1/2	163 (52.41)
≤ 3/4	65 (20.90)
≤ 7/8	41 (13.18)
≤ 1	31 (9.97)

ESTD: Endoscopic submucosal tunnel dissection.

Table 2 Pre-endoscopic submucosal tunnel dissection and post-endoscopic submucosal tunnel dissection pathology n (%)

Pathology	Pre-ESTD	Post-ESTD	Pre-ESTD and Post-ESTD coincidence
Inflammation	0	3 (0.96)	0
LGIN	67 (21.54)	43 (13.83)	36 (11.57)
HGIN	159 (51.13)	52 (16.72)	37 (11.90)
M1	74 (23.79)	74 (23.79)	18 (5.79)
M2	11 (3.54)	47 (15.11)	3 (0.96)
M3	0	51 (16.40)	0
SM1	0	23 (7.40)	0
> SM1	0	18 (5.79)	0

ESTD: Endoscopic submucosal tunnel dissection; LGIN: Low-grade intraepithelial neoplasia; HGIN: High-grade intraepithelial neoplasia; M1: Carcinoma in situ; M2: Carcinoma infiltrated to laminae propria; M3: Carcinoma infiltrated to muscularis mucosae; SM1: Submucosal invasion < 200 μm; SM2: Submucosal invasion > 200 μm.

version 24.0, SPSS Inc, Armonk, NY, United States). *P*-value < 0.05 indicated statistical significance.

RESULTS

Baseline characteristics of patients

A total of 355 superficial esophageal patients underwent endoscopic treatments from March 1, 2013 to May 1, 2017, of which 66 patients were excluded for the following reasons: (1) Adenocarcinoma (*n* = 15); (2) EMR procedure (*n* = 10) or ESD procedure (*n* = 27); (3) incomplete lesion data (*n* = 6); and (4) lost to follow-up (*n* = 8), as shown in Figure 1. The demographic data

are shown in Table 1. The average age of the patients was 61.39 ± 8.07 years with a male/female ratio of 2.17 (213/98). The lesions were mainly located in the middle third of the esophagus (64.31%). Thirty-one circumferential lesions were included in the final analysis (Table 1). The most common preoperative histological type was HGIN, as shown in Table 2.

Treatment outcomes and complications

Three hundred eleven lesions were successfully resected from 289 patients. The average specimen area was 14.1 ± 3.6 cm^2, the mean procedure time was 102.4 ± 35.3 min, and the mean dissection speed was 18.6 ± 2.1 mm^2/min. A total of 308 (308/311) lesions were resected by en bloc (99.04%), of which 49 were diagnosed with horizontal or vertical margin involvement by pathological evaluation; thus, the R0 resection rate was 81.28%

Table 3 Endoscopic submucosal tunnel dissection procedure characteristics *n* (%)

Category	ESTD (*n* = 311)
Specimen area, cm², mean ± SD	14.1 ± 3.6
Tumor width diameter, cm, mean ± SD	3.1 ± 0.6
Tumor length diameter, cm, mean ± SD	4.2 ± 0.9
Procedure time, min, mean ± SD	102.4 ± 35.3
Dissection speed, mm²/ min, mean ± SD	18.6 ± 2.1
En bloc resection	308 (99.04)
R0 resection	259 (81.28)
Curative resection	244 (78.46)
Hospitalization day, d, mean ± SD	10.3 ± 2.8
Hospitalization expense, dollars, mean ± SD	3766.5 ± 846.5

ESTD: Endoscopic submucosal tunnel dissection.

Table 4 Endoscopic submucosal tunnel dissection-related complications *n* (%)

Category	ESTD (*n* = 311)
Post-operative infection	30 (9.65)
Bleeding	
Intraoperative bleeding	20 (6.43)
Postoperative bleeding	5 (1.61)
Muscular injury	98 (31.51)
Perforation	6 (1.93)
Esophageal stricture	46 (14.79)
Positive margin	
Horizontal margin	35 (11.25)
Vertical margin	10 (3.22)
Horizontal and vertical margin	4 (1.29)
Lymphovascular invasion	12 (3.86)

ESTD: Endoscopic submucosal tunnel dissection.

(259/311). Twelve patients were diagnosed with lymphovascular invasion (3.86%), of which five were combined with positive margin. We evaluated the invasion depth under microscopy and observed that seven lesions had a submucosal invasion deeper than 200 μm. As a result, the curative resection rate was 78.46% (244/311). After post-ESTD pathological evaluation, three patients were diagnosed with residual cancer in horizontal margin and 12 in vertical margin, of which five patients had vascular invasion. Another seven patients simply showed vascular invasion. All of the 22 patients were recommended an additional surgery. Finally, 17 patients underwent surgery, while the other five refused and were closely observed. The mean hospitalization stay was 10.3 ± 2.8 d, while the average hospitalization expense was 3766.5 ± 846.5 dollars (Table 3).

After ESTD procedure, 30 patients had postoperative infection, of which 29 were pulmonary infection, and one was urinary-tract infection. All of the infections were cured by intravenous infusion of antibiotics. Moreover, 20 (6.43%) patients had intra-operative bleeding, and five patients had postoperative bleeding. All of these patients underwent endoscopic hemostasis, and no severe complications with regard to bleeding were observed. Six patients had esophageal perforation and were cured by conservative treatment. Forty-six patients had postoperative esophageal stricture, of which 36 (78.26%) underwent an average of 4.1 (2-19 times) endoscopic balloon dilations in a mean follow-up time of 20.2 mo. In addition, the dysphagia was almost relieved (Table 4), while the other 10 patients with obstinate stenosis were further managed by receiving endoscopic balloon dilatation every 2 wk till dysphagia was relieved.

Pathology analysis
We analyzed the pathological change between pre-ESTD biopsies and post-ESTD specimens and observed that HGIN accounted for 51.13% (159/311) of pre-ESTD biopsies, while in post-ESTD pathology, superficial invasive carcinoma accounted for 44.70% (139/311). The pre-ESTD and post-ESTD coincidence rate was 30.23% (94/311). Also, 50.21% (117/233) of HGIN and M1 lesions had a pathological upgrade after ESTD to superficial invasive carcinoma.

DISCUSSION

This study evaluated the efficacy and complications of ESTD in 289 patients with 311 esophageal mucosal lesions. ESTD is a new technique developed from ESD and tunnel endoscopy. There are currently few studies that have reported the efficacy and complications of ESTD in large samples. Gan *et al*[13] reported endoscopic submucosal multi-tunnel dissection (ESMTD) for seven circumferential lesions, in which all patients achieved R0 resection but suffered from esophageal stricture. Huang *et al*[10] compared the efficacy and complication rate between ESD and ESTD using a propensity score matching analysis and observed that ESTD can improve procedure efficacy and reduce injury to muscular layer due to a better view, more efficient vessel coagulation, and longer lasting submucosal liquid cushion. In our previous ESD procedure, we observed that the dissected mucosa shrank and blocked the lumen, making it difficult to obtain a clear view. While ESTD can avoid this obstacle by creating a submucosal tunnel, when therapeutic endoscopy enters the submucosal tunnel, it will acquire a clear operative view to facilitate observation of the submucosal vessels and muscular layer, thereby reducing the bleeding and perforation rate. For this reason, it is especially appropriate for large mucosal lesions[11]. Zhai *et al*[19] obtained similar findings and noted that ESTD is indicated when (1) lesions do not invade deeper than sm1 and have no evidence of lymph node metastasis and (2) the lesion's circumference level ≥ 1/3 or the diameter ≥ 2 cm.

The reported en bloc and R0 resection rates of ESTD were 97.8% (92%-100%) and 85.6%（81.8%-100%）, which are similar to our study outcomes. However, our curative resection rate was 78.46% (244/311), mainly because we included large mucosal lesions. Also, 44.05% (137/311) of our lesions had a circumference level > 1/2, which may increase the risk of incomplete resection.

Patients' mean hospitalization stay was 10.3 ± 2.8 d, which is closely related to less hospitalization expenses (3766.5 ± 846.5 dollars) compared with surgical treatment.

We evaluated the post-ESTD specimens' pathological features and observed that 50.21% (117/233) of HGIN and M1 lesions upgraded to superficial invasive carcinoma after ESTD. Several reasons might contribute to this: First, the heavier the lesion and the wider its range, the poorer the representativeness of the pre-ESTD biopsy. In large or multifocal lesions, even if multiple biopsies are taken, it is difficult to represent the whole picture of the lesion. Moreover, the esophagus wall is thin; thus, too deeply drawn or frequent biopsies will lead to bleeding, perforation, and other biopsy-related complications. Therefore, we think that the reference significance of preoperative biopsy requires further evaluation and should be combined with iodine staining, narrow band imaging with magnifying endoscopy (ME-NBI), and radiological examination.

Postoperative infection, bleeding, and perforation are common in ESD procedure. Previous studies reported the bleeding and perforation rates of ESD to be 0-6% and 1.7%-4.0%[20-22], respectively. In our study, the total bleeding rate and perforation rate associated with ESTD were 8.04% and 1.93%, respectively. The significant bleeding that needs postoperative hemostatic treatment is relatively low (1.61%), indicating that ESTD is a safe treatment method for superficial esophageal squamous cell carcinoma and precancerous lesions. Thirty (9.65%) patients had postoperative infection. There are no available studies that have reported on post-ESD infection, although it is relatively common especially for the elderly. We speculate that the infection is caused by the patient hypoimmunity and the history of previous pulmonary disease or inhalation pneumonia related to anesthesia; however, further studies are needed to confirm this etiology.

Esophageal stricture and positive margin are serious complications of ESTD procedure, and the incidence rates found in our study were 14.79% (46/311) and 15.76% (49/311), respectively. It was reported that the circumference level and the area of the lesion are risk factors for esophageal stricture[23,24]. The incidence rate of esophageal stricture in patients with circumference level > 3/4 is above 70%-90%[25,26]. When the lesion area is large enough, the artificial esophageal ulcer causes excess absence of epithelial cells and results in fibrous repair in the submucosa[27], which is the primary cause of esophageal stricture. To prevent esophageal stricture, the administration of steroids is useful, as previously reported[28,29], while endoscopic balloon dilation and esophageal stent implantation can also be options[30,31]. For positive margin, previous studies reported its incidence after ESD to be 3%-17%[18,32,33]. Wen reported that the lesion area and invasion depth are risk factors of positive margin[33], and hypothesized that a greater lesion area and deeper invasion level corresponded to higher positive rates of the incisal margin. When treating large and multiple lesions, the risk of positive margin is relatively high, and thus accurate preoperative labeling and intraoperative complete resection are important. There are no standard guidelines to address positive margin after endoscopic resection; therefore, we recommended additional surgery for all patients in our study with positive basal margin, horizontal margin carcinoma involvement, and vascular invasion; however, several patients refused and entered the follow-up cohort. No residual or recurrent tumor was observed during the follow-up period.

The present study is the largest sample research of ESTD technique to date, and our observation indicators are complete, the follow-up period is long, the results are credible, and there is strong reference significance in clinical work. Moreover, our study also performed a detailed evaluation of postoperative pathology and emphasized its guiding role in the postoperative management of the patients. However, this study has several limitations. Firstly, this was a retrospective study and thus has inherent case selection bias. Secondly, this was a single center study; therefore, the operation level of ESTD in this study cannot be fully represented in whole.

In conclusion, ESTD for superficial esophageal squamous cell carcinoma and precancerous lesions is effective and safe, exhibiting high en bloc resection rate as well as low bleeding and perforation rates. When using ESTD to resect large mucosal lesions, the incidence of postoperative esophageal stricture and positive margin is high, and thus other effective preventative measures should be considered. We also observed that preoperative biopsies cannot represent the whole specimen, while half of the biopsies' pathology upgraded after ESTD procedure; therefore, the choice of therapy cases should be made cautiously before ESTD.

ARTICLE HIGHLIGHTS

Research background
Endoscopic submucosal dissection (ESD) is becoming the standard treatment for early gastrointestinal cancers, as it has a higher en bloc resection rate and a lower recurrence rate than endoscopic mucosal resection (EMR). However, when treating large mucosal lesions, ESD always faces many difficulties, such as multiple submucosal injections times, long procedure time, and low complete resection rate. To overcome these difficulties, a modified technique named endoscopic submucosal tunnel dissection (ESTD) has been proposed. Compared with ESD, ESTD could reduce the number of submucosal injections, shorten the procedure time, increase the resection speed, and reduce the injury of the muscular layer. However, there are no studies that verify the feasibility of ESTD in superficial esophageal squamous cell carcinoma (ESCC) and precancerous lesions in a large sample.

Research motivation
To our knowledge, the present study is the largest sample research of ESTD technique to date, and our observation indicators are complete, the follow-up period is long, the results are credible, and there is strong reference significance in clinical work.

Research objectives
This study aims to evaluate the clinical outcomes of patients who underwent ESTD for ESCC and precancerous lesions.

Research methods

ESTD was performed in 289 patients with 311 lesions. The clinical outcomes of the patients and pathological features of the lesions were retrospectively reviewed.

Research results

A total of 311 lesions were included. The en bloc rate, complete resection rate, and curative resection rate were 99.04%, 81.28%, and 78.46%, respectively. The ESTD procedure time was 102.4 ± 35.1 min, the mean hospitalization time was 10.3 ± 2.8 d, and the average expenditure was 3766.5 ± 846.5 dollars. The intraoperative bleeding rate, postoperative bleeding rate, the perforation rate, and the postoperative infection rate were 6.43%, 1.61%, 1.93%, and 9.65%, respectively. Esophageal stricture and positive margin were severe adverse events, with an incidence rate of 14.79% and 15.76%, respectively. No tumor recurrence occurred during the follow-up period.

Research conclusions

ESTD for ESCC and precancerous lesions is feasible and relatively safe, but the rates of esophageal stricture and positive margin are high for large mucosal lesions.

Research perspectives

The present study is a retrospective study to describe the general characteristics of ESTD. In the future, case control studies and prospective studies are considered necessary to evaluate further the feasibility and safety of ESTD for treating ESCC and precancerous lesions.

REFERENCES

1   **Lian J**, Chen S, Zhang Y, Qiu F. A meta-analysis of endoscopic submucosal dissection and EMR for early gastric cancer. *Gastrointest Endosc* 2012; **76**: 763-770 [PMID: 22884100 DOI: 10.1016/j.gie.2012.06.014]

2   **Ishihara R**, Iishi H, Uedo N, Takeuchi Y, Yamamoto S, Yamada T, Masuda E, Higashino K, Kato M, Narahara H, Tatsuta M. Comparison of EMR and endoscopic submucosal dissection for en bloc resection of early esophageal cancers in Japan. *Gastrointest Endosc* 2008; **68**: 1066-1072 [PMID: 18620345 DOI: 10.1016/j.gie.2008.03.1114]

3   **Pimentel-Nunes P**, Dinis-Ribeiro M, Ponchon T, Repici A, Vieth M, De Ceglie A, Amato A, Berr F, Bhandari P, Bialek A, Conio M, Haringsma J, Langner C, Meisner S, Messmann H, Morino M, Neuhaus H, Piessevaux H, Rugge M, Saunders BP, Robaszkiewicz M, Seewald S, Kashin S, Dumonceau JM, Hassan C, Deprez PH. Endoscopic submucosal dissection: European Society of Gastrointestinal Endoscopy (ESGE) Guideline. *Endoscopy* 2015; **47**: 829-854 [PMID: 26317585 DOI: 10.1055/s-0034-1392882]

4   **Maeda Y**, Hirasawa D, Fujita N, Obana T, Sugawara T, Ohira T, Harada Y, Yamagata T, Suzuki K, Koike Y, Yamamoto Y, Kusaka Z, Noda Y. A pilot study to assess mediastinal emphysema after esophageal endoscopic submucosal dissection with carbon dioxide insufflation. *Endoscopy* 2012; **44**: 565-571 [PMID: 22407383 DOI: 10.1055/s-0031-1291664]

5   **Linghu E**, Feng X, Wang X, Meng J, Du H, Wang H. Endoscopic submucosal tunnel dissection for large esophageal neoplastic lesions. *Endoscopy* 2013; **45**: 60-62 [PMID: 23254407 DOI: 10.1055/s-0032-1325965]

6   **Tsao SK**, Toyonaga T, Morita Y, Fujita T, Hayakumo T, Azuma T. Modified fishing-line traction system in endoscopic submucosal dissection of large esophageal tumors. *Endoscopy* 2011; **43** Suppl 2: E119 [PMID: 21425004 DOI: 10.1055/s-0030-1256148]

7   **Xie X**, Bai JY, Fan CQ, Yang X, Zhao XY, Dong H, Yang SM, Yu J. Application of clip traction in endoscopic submucosal dissection to the treatment of early esophageal carcinoma and precancerous lesions. *Surg Endosc* 2017; **31**: 462-468 [PMID: 27126625 DOI: 10.1007/s00464-016-4939-1]

8   **Koike Y**, Hirasawa D, Fujita N, Maeda Y, Ohira T, Harada Y, Suzuki K, Yamagata T, Tanaka M. Usefulness of the thread-traction method in esophageal endoscopic submucosal dissection: randomized controlled trial. *Dig Endosc* 2015; **27**: 303-309 [PMID: 25357187 DOI: 10.1111/den.12396]

9   **Linghu E**. Therapeutics of Digestive Endoscopic Tunnel Technique. Berlin, Springer Netherlands, 2014 [DOI: 10.1007/978-94-007-7344-8]

10 **Huang R**, Cai H, Zhao X, Lu X, Liu M, Lv W, Liu Z, Wu K, Han Y. Efficacy and safety of endoscopic submucosal tunnel dissection for superficial esophageal squamous cell carcinoma: a propensity score matching analysis. *Gastrointest Endosc* 2017; **86**: 831-838 [PMID: 28286094 DOI: 10.1016/j.gie.2017.03.001]

11 **Wang J**, Qin JY, Guo TJ, Gan T, Wang YP, Wu JC. The Efficiency and Complications of ESD and ESTD in the Treatment of Large Esophageal Mucosal Lesions. *Sichuan Da Xue Xue Bao Yi Xue Ban* 2015; **46**: 896-900 [PMID: 26867327]

12 **Zhai Y**, Linghu E, Li H, Qin Z, Feng X, Wang X, Du H, Meng J, Wang H, Zhu J. [Comparison of endoscopic submucosal tunnel dissection with endoscopic submucosal dissection for large esophageal superficial neoplasms]. *Nan Fang Yi Ke Da Xue Xue Bao* 2014; **34**: 36-40 [PMID: 24463113]

13 **Gan T**, Yang JL, Zhu LL, Wang YP, Yang L, Wu JC. Endoscopic submucosal multi-tunnel dissection for circumferential superficial esophageal neoplastic lesions (with videos). *Gastrointest Endosc* 2016; **84**: 143-146 [PMID: 26828761 DOI: 10.1016/j.gie.2016.01.049]

14 **Japan Esophageal Society.**. Japanese Classification of Esophageal Cancer, 11th Edition: part I. *Esophagus* 2017; **14**: 1-36 [PMID: 28111535 DOI: 10.1007/s10388-016-0551-7]

15 . The Paris endoscopic classification of superficial neoplastic lesions: esophagus, stomach, and colon: November 30 to December 1, 2002. *Gastrointest Endosc* 2003; **58**: S3-S43 [PMID: 14652541 DOI: 10.1016/S0016-5107(03)02159-X]

16 **Loizou LA**, Grigg D, Atkinson M, Robertson C, Bown SG. A prospective comparison of laser therapy and intubation in endoscopic palliation for malignant dysphagia. *Gastroenterology* 1991; **100**: 1303-1310 [PMID: 1707386 DOI: 10.1016/0016-5085(91)90782-G]

17 **Qumseya BJ**, Wolfsen C, Wang Y, Othman M, Raimondo M, Bouras E, Wolfsen H, Wallace MB, Woodward T. Factors associated with increased bleeding post-endoscopic mucosal resection. *J Dig Dis* 2013; **14**: 140-146 [PMID: 23134152 DOI: 10.1111/1751-2980.12002]

18 **Joo DC**, Kim GH, Park DY, Jhi JH, Song GA. Long-term outcome after endoscopic submucosal dissection in patients with superficial esophageal squamous cell carcinoma: a single-center study. *Gut Liver* 2014; **8**: 612-618 [PMID: 25368748 DOI: 10.5009/gnl13130]

19 **Zhai YQ**, Li HK, Linghu EQ. Endoscopic submucosal tunnel dissection for large superficial esophageal squamous cell neoplasms. *World J Gastroenterol* 2016; **22**: 435-445 [PMID: 26755889 DOI: 10.3748/wjg.v22.i1.435]

20 **Oyama T**, Tomori A, Hotta K, Morita S, Kominato K, Tanaka M, Miyata Y. Endoscopic submucosal dissection of early esophageal cancer. *Clin Gastroenterol Hepatol* 2005; **3**: S67-S70 [PMID: 16013002 DOI: 10.1016/S1542-3565(05)00291-0]

21 **Ono S**, Fujishiro M, Niimi K, Goto O, Kodashima S, Yamamichi N, Omata M. Long-term outcomes of endoscopic submucosal dissection for superficial esophageal squamous cell neoplasms. *Gastrointest Endosc* 2009; **70**: 860-866 [PMID: 19577748 DOI: 10.1016/j.gie.2009.04.044]

22 **Sohara N**, Hagiwara S, Arai R, Iizuka H, Onozato Y, Kakizaki S. Can endoscopic submucosal dissection be safely performed in a smaller specialized clinic? *World J Gastroenterol* 2013; **19**: 528-535 [PMID: 23382632 DOI: 10.3748/wjg.v19.i4.528]

23 **Ono S**, Fujishiro M, Niimi K, Goto O, Kodashima S, Yamamichi N, Omata M. Predictors of postoperative stricture after esophageal endoscopic submucosal dissection for superficial squamous cell neoplasms. *Endoscopy* 2009; **41**: 661-665 [PMID: 19565442 DOI: 10.1055/s-0029-1214867]

24 **Katada C**, Muto M, Manabe T, Boku N, Ohtsu A, Yoshida S. Esophageal stenosis after endoscopic mucosal resection of superficial esophageal lesions. *Gastrointest Endosc* 2003; **57**: 165-169 [PMID: 12556777 DOI: 10.1067/mge.2003.73]

25   **Ezoe Y**, Muto M, Horimatsu T, Morita S, Miyamoto S, Mochizuki S, Minashi K, Yano T, Ohtsu A, Chiba T. Efficacy of preventive endoscopic balloon dilation for esophageal stricture after endoscopic resection. *J Clin Gastroenterol* 2011; **45**: 222-227 [PMID: 20861798 DOI: 10.1097/MCG.0b013e3181f39f4e]

26   **Hashimoto S**, Kobayashi M, Takeuchi M, Sato Y, Narisawa R, Aoyagi Y. The efficacy of endoscopic triamcinolone injection for the prevention of esophageal stricture after endoscopic submucosal dissection. *Gastrointest Endosc* 2011; **74**: 1389-1393 [PMID: 22136782 DOI: 10.1016/j.gie.2011.07.070]

27   **Honda M**, Nakamura T, Hori Y, Shionoya Y, Yamamoto K, Nishizawa Y, Kojima F, Shigeno K. Feasibility study of corticosteroid treatment for esophageal ulcer after EMR in a canine model. *J Gastroenterol* 2011; **46**: 866-872 [PMID: 21597933 DOI: 10.1007/s00535-011-0400-3]

28   **Ratone JP**, Bories E, Caillol F, Pesenti C, Godat S, Poizat F, Cassan C, Giovannini M. Oral steroid prophylaxis is effective in preventing esophageal strictures after large endoscopic resection. *Ann Gastroenterol* 2017; **30**: 62-66 [PMID: 28042239 DOI: 10.20524/aog.2016.0085]

29   **Yamaguchi N**, Isomoto H, Nakayama T, Hayashi T, Nishiyama H, Ohnita K, Takeshima F, Shikuwa S, Kohno S, Nakao K. Usefulness of oral prednisolone in the treatment of esophageal stricture after endoscopic submucosal dissection for superficial esophageal squamous cell carcinoma. *Gastrointest Endosc* 2011; **73**: 1115-1121

[PMID: 21492854 DOI: 10.1016/j.gie.2011.02.005]

30   **Sato H**, Inoue H, Kobayashi Y, Maselli R, Santi EG, Hayee B, Igarashi K, Yoshida A, Ikeda H, Onimaru M, Aoyagi Y, Kudo SE. Control of severe strictures after circumferential endoscopic submucosal dissection for esophageal carcinoma: oral steroid therapy with balloon dilation or balloon dilation alone. *Gastrointest Endosc* 2013; **78**: 250-257 [PMID: 23453294 DOI: 10.1016/j.gie.2013.01.008]

31   **Yano T**, Yoda Y, Nomura S, Toyosaki K, Hasegawa H, Ono H, Tanaka M, Morimoto H, Horimatsu T, Nonaka S, Kaneko K, Sato A. Prospective trial of biodegradable stents for refractory benign esophageal strictures after curative treatment of esophageal cancer. *Gastrointest Endosc* 2017; **86**: 492-499 [PMID: 28137598 DOI: 10.1016/j.gie.2017.01.011]

32   **Repici A**, Hassan C, Carlino A, Pagano N, Zullo A, Rando G, Strangio G, Romeo F, Nicita R, Rosati R, Malesci A. Endoscopic submucosal dissection in patients with early esophageal squamous cell carcinoma: results from a prospective Western series. *Gastrointest Endosc* 2010; **71**: 715-721 [PMID: 20363414 DOI: 10.1016/j.gie.2009.11.020]

33   **Wen J**, Linghu E, Yang Y, Liu Q, Wang X, Du H, Wang H, Meng J, Lu Z. Relevant risk factors and prognostic impact of positive resection margins after endoscopic submucosal dissection of superficial esophageal squamous cell neoplasia. *Surg Endosc* 2014; **28**: 1653-1659 [PMID: 24380990 DOI: 10.1007/s00464-013-3366-9]

ORIGINAL ARTICLE

Retrospective Study

Impact of the number of examined lymph nodes on outcomes in patients with lymph node-negative gallbladder carcinoma

Dong-Xu Fan, Rong-Wei Xu, Yong-Cheng Li, Bing-Qing Zhao, Ming-Yu Sun

Dong-Xu Fan, Department of Vascular surgery, First Affiliated Hospital of Jiamusi University, Jiamusi 154002, Heilongjiang Province, China

Rong-Wei Xu, Department of Vascular Surgery, Shandong Provincial Qianfoshan Hospital, Shandong University, Jinan 250014, Shandong Province, China

Yong-Cheng Li, Department of Medical Oncology, Xuzhou Central Hospital, The Affiliated Xuzhou Hospital of Medical College of Southeast University, Xuzhou 221009, Jiangsu Province, China

Bing-Qing Zhao, Department of Surgery, Tianjin Second People's Hospital, Tianjin 300192, China

Ming-Yu Sun, Department of Breast Surgery, Xuzhou Central Hospital, The Affiliated Xuzhou Hospital of Medical College of Southeast University, Xuzhou 221009, Jiangsu Province, China

ORCID number: Dong-Xu Fan (0000-0003-0390-231X); Rong-Wei Xu (0000-0002-4075-8559); Yong-Cheng Li (0000-0002-4359-7439); Bing-Qing Zhao (0000-0001-5248-7074); Ming-Yu Sun (0000-0003-2172-5118).

Author contributions: Fan DX and Sun MY designed the research and critically revised the manuscript for important intellectual content; all authors performed the research, analyzed the data, and wrote the paper.

Conflict-of-interest statement: All authors declare no competing financial interests.

Abstract

AIM

To determine whether the number of examined lymph nodes (LNs) is correlated with the overall survival of gallbladder carcinoma (GBC) patients.

METHODS

Patients were collected from the Surveillance Epidemiology and End Results database (2004-2013) and categorized by the number of LNs into six groups: 1 LN, 2 LNs, 3 LNs, 4 LNs, 5 LNs, and ⩾ 6 LNs. Survival curves for overall survival were plotted with a Kaplan-Meier analysis. The log-rank test was used for univariate comparisons.

RESULTS

In a cohort of 893 patients, the median number of examined LNs was two for the entire cohort. The survival for the 1 LN group was significantly poorer than those of the stage Ⅰ and Ⅱ disease groups and for the entire

cohort. By dichotomizing the number of LNs from 1 to 6, we found that the minimum number of LNs that should be examined was four for stage Ⅰ, four or five for stage Ⅱ, and six for stage ⅢA disease. Therefore, for the entire cohort, the number of examined LNs should be at least six, which is exactly consistent with the American Joint Committee on Cancer criteria.

CONCLUSION
The examination of higher numbers of LNs is associated with improved survival after resection surgery for N0 GBC. The guidelines for GBC surgery, which recommend that six LNs be examined at least, are statistically valid and should be applied in clinical practice widely.

Key words: Gallbladder carcinoma; Lymph node; N0 stage; Prognostic factor

Core tip: Six lymph nodes were recommended as the minimum number of examination in the 8th edition American Joint Committee on Cancer tumor-node-metastasis criteria for gallbladder carcinoma, but the rationality has not been evaluated yet. Thus, we aimed to explore the optimal lymph node number using the Surveillance Epidemiology and End Results database.

INTRODUCTION

Gallbladder carcinoma (GBC) is one of the most lethal carcinomas and has a poor prognosis[1-3]. To date, surgery remains the only radical treatment strategy for patients, translating into 5-year survival rates of approximately 5%[4-7]. Lymph node (LN) status is an important prognostic factor for GBC patients[8]. Unfortunately, LN metastases occur in more than 50% of patients, and LN-positive patients are widely known to have very poor survival[4].

The role of regional and extended lymphadenectomy for GBC has been previously investigated[9-12], but there is not a general consensus about the number of LNs that should be examined. In the 8th edition of the tumor-node-metastasis (TNM) staging system for GBC from the American Joint Committee on Cancer (AJCC), the N category was defined by the number of metastatic LNs instead of the location of the metastatic LNs, as used in the previous edition, and was correlated with prognosis. These guidelines recommend examining a minimum of six LNs to accurately classify patients with GBC[13]. Thus, this study aimed to assess patients with LN-negative (N0) GBC to determine whether the number of examined LNs was correlated with overall survival of GBC patients. We used the Surveillance, Epidemiology, and End Result (SEER) database to determine the influence of the number of examined LNs on prognosis in patients with N0 GBC.

MATERIALS AND METHODS

Patients
The SEER database (2004-2013) was used to identify patients with GBC. Patients who met the following criteria were included: (1) Pathologically confirmed diagnosis; (2) radical surgical treatment; (3) definite cancer stage according to the 8th edition of the AJCC criteria; (4) first primary tumor; (5) number of positive LNs equal to zero; (6) no distant metastases; (7) one or more LNs examined; and (8) active follow-up. The exclusion criteria were as follows: (1) Age < 18 years; (2) unavailable follow-up data or 0 d of follow-up; (3) unknown cause of death; (4) number of LNs examined coded with SEER codes 95 to 99 (the information about the number of LN is not available); and (5) T4 disease.

Statistical analysis
The clinicopathological characteristics were compared among the stage Ⅰ, Ⅱ, and ⅢA disease subgroups by the independent *t* test for continuous variables and the chi-square test for categorical variables. Overall survival (OS) was determined from the SEER record of survival time (total number of months) and vital status. The relationship between the number of examined LNs and OS was assessed separately for the entire cohort and for stage Ⅰ, Ⅱ, and ⅢA patients. Patients were categorized by the number of examined LNs into the following six groups: 1 LN, 2 LNs, 3 LNs, 4 LNs, 5 LNs, and ≥ 6 LNs. The optimal number of examined LNs was determined with X-tile software (Yale University, Version 3.6.1). Survival curves for OS were plotted with a Kaplan-Meier analysis. The log-rank test was used for univariate comparison. A Cox proportional hazard method was used to identify factors associated with mortality and to calculate hazard ratios (HRs) and 95% confidence intervals (CIs). The variables, including age, sex, race, radiation therapy, number of examined LNs, grade, and stage, that were significant in univariate analysis, were included in the Cox model. All the statistical analyses were performed using SPSS, version 20 (Armonk, NY, United States). A two-tailed *P*-value of < 0.05 was considered statistically significant.

RESULTS

Among the 893 patients who were finally eligible for this analysis, 228 patients (25.5%) had stage Ⅰ disease, 444 patients (49.7%) had stage Ⅱ disease, and 221

Table 1 Demographic and tumor characteristics for patients with lymph node-negative gallbladder carcinoma *n* (%)

Characteristics	Entire cohort *n* = 893 (100%)	Stage I [1] *n* = 228 (25.5%)	Stage II [1] *n* = 444 (49.7%)	Stage IIIA[1] *n* = 221 (24.7%)	*P* [2] Value
Age, yr					
Median (range)	67 (21-96)	67 (21-92)	67 (25-96)	67 (35-93)	0.575
Sex					
Male	272 (30.5)	63 (27.6)	139 (31.3)	70 (31.7)	
Female	621 (69.5)	165 (72.4)	305 (68.7)	151 (68.3)	0.559
Race					
White	675 (75.6)	162 (71.1)	34.1 (76.8)	172 (77.8)	
Black	106 (11.9)	33 (14.5)	50 (11.3)	23 (10.4)	
Others	112 (12.5)	33 (14.5)	53 (11.9)	26 (11.8)	0.261
Grade[1]					
Well	187 (20.9)	67 (29.4)	102 (23.0)	18 (8.1)	
Moderate	414 (46.4)	101 (44.3)	215 (48.4)	98 (44.3)	
Poor	210 (23.5)	26 (11.4)	96 (21.6)	88 (39.8)	
Undifferentiated	11 (1.2)	1 (0.4)	5 (1.1)	5 (2.3)	
Unknown	71 (8.0)	33 (14.5)	26 (5.9)	12 (5.4)	< 0.001
Radiation					
Yes	151 (16.9)	8 (3.5)	75 (16.9)	68 (30.8)	
No	742 (83.1)	220 (96.5)	369 (83.1)	153 (69.2)	< 0.001
Vital status					
Alive	547 (61.3)	151 (66.2)	308 (69.4)	88 (39.8)	
Dead	346 (38.7)	77 (33.8)	136 (30.6)	133 (60.2)	< 0.001
Number of LN					
Median (range)	2 (1-80)	1 (1-80)	2 (1-40)	2 (1-24)	0.590

[1]AJCC/TNM 8th edition. [2]Factors were compared by the independent t test and the χ^2 test for continuous and categorical variables, respectively. LN: Lymph node.

Figure 1 Number of examined lymph nodes was categorized into subgroups. The bars in the graph reflect the percentage of patients who fell into each subgroup. Entire cohort, *n* = 893; stage I, *n* = 228; stage II, *n* = 444; stage IIIA, *n* = 221.

patients (24.7%) had stage IIIA disease. The median age at diagnosis for the entire cohort was 67 years (range 21-96 years), and 272 patients (30.5%) were male.

The clinical characteristics of the entire cohort and patients with stage I, II, and IIIA disease are listed in Table 1. There was no difference among patients with stage I, II, or IIIA disease in terms of age, sex, race, and number of LNs examined. In addition, compared with patients with stage I and II disease, a larger proportion of patients with stage IIIA disease had poor/undifferentiated tumors and received radiation therapy.

The median number of examined LNs was 2 for the entire cohort, 1 LN for the stage I group, 2 LNs for the stage II group, and 2 LNs for the stage IIIA group. More than 40% of the patients had only 1 LN examined, and a lower proportion of patients had more LNs examined (Figure 1). The number of examined LNs did not differ by stage (*P* = 0.59).

Patients were categorized by the number of examined LNs into the following 6 groups: 1 LN, 2 LNs, 3 LNs, 4 LNs, 5 LNs and ≥ 6 LNs. Survival in relation to the number of examined LNs was assessed separately for the entire cohort and patients with stage I, II and IIIA disease (Table 2). For the entire cohort, a median survival of 18 mo and a 5-year survival rate of 0.393 were noted for patients with one LN examined (*n* = 398). The survival for the 1 LN group was significantly poorer than that of the other groups (*P* < 0.001, Figure 2). However, there was no difference in survival among the other five groups (*P* > 0.05). For patients with stage I disease, the median survival for the 1 LN, 2 LNs, 3 LNs, 4 LNs, 5 LNs, and ≥ 6 LNs groups was 24, 43, 30, 22, 38, and 26 mo, respectively. Similar survival results according to the LN groups were demonstrated for patients with stage I and II disease but not for patients with stage IIIA disease (Table 2). However, compared with patients with stage I and II disease, the median survival and 5-year survival rate of patients with stage IIIA disease was obviously decreased in all the LN groups. For example, the 5-year survival rate in the 1 LN group was 0.473 for stage I, 0.445 for stage II, and 0.177 for stage IIIA disease. As shown in Table 3, there was no difference in

Table 2 Survival by lymph node group and stage

Number of LN	Entire cohort *n* = 893 (100%)	Stage I [1] *n* = 228 (25.5%)	Stage II [1] *n* = 444 (49.7%)	Stage IIIA [1] *n* = 221 (24.7%)
1 LN				
Patients (*n*)	398	121	189	88
Median OS, mo	18	24	20	10
3-yr SR (95%CI)	0.503 (0.474-0.532)	0.603 (0.553-0.653)	0.608 (0.566-0.650)	0.271 (0.217-0.325)
5-yr SR (95%CI)	0.393 (0.361-0.425)	0.473 (0.418-0.528)	0.445 (0.394-0.496)	0.177 (0.128-0.226)
2 LNs				
Patients (*n*)	129	35	68	26
Median OS, mo	28	43	27	15
3-yr SR (95%CI)	0.711 (0.665-0.757)	0.808 (0.737-0.879)	0.775 (0.713-0.837)	0.425 (0.317-0.533)
5-yr SR (95%CI)	0.579 (0.524-0.634)	0.725 (0.640-0.810)	0.586 (0.504-0.668)	0.340 (0.225-0.455)
3 LNs				
Patients (*n*)	85	20	33	32
Median OS, mo	21	30	27	11
3-yr SR (95%CI)	0.587 (0.525-0.649)	0.722 (0.603-0.841)	0.697 (0.606-0.788)	0.379 (0.277-0.481)
5-yr SR (95%CI)	0.466 (0.396-0.536)	0.602 (0.454-0.750)	0.639 (0.539-0.739)	0.203 (0.109-0.297)
4 LNs				
Patients (*n*)	55	8	31	16
Median OS, mo	22	22	27	10
3-yr SR (95%CI)	0.638 (0.560-0.716)	0.833 (0.681-0.985)	0.760 (0.671-0.849)	0.295 (0.154-0.446)
5-yr SR (95%CI)	0.533 (0.438-0.628)	0.833 (0.681-0.985)	0.652 (0.526-0.778)	0.148 (0.022-0.274)
5 LNs				
Patients (*n*)	43	10	21	12
Median OS, mo	24	38	24	18
3-yr SR (95%CI)	0.750 (0.671-0.829)	0.857 (0.725-0.989)	NA	0.292 (0.133-0.451)
5-yr SR (95%CI)	0.652 (0.557-0.747)	0.714 (0.543-0.885)	0.857 (0.725-0.989)	0.146 (0.016-0.276)
≥ 6 LNs				
Patients (*n*)	183	34	102	47
Median OS, mo	26	26	26	21
3-yr SR (95%CI)	0.696 (0.655-0.737)	0.817 (0.731-0.903)	0.753 (0.701-0.805)	0.498 (0.412-0.584)
5-yr SR (95%CI)	0.594 (0.547-0.641)	0.817 (0.731-0.903)	0.671 (0.610-0.732)	0.306 (0.221-0.391)

[1]AJCC/TNM 8th edition. LN: Lymph node; OS: Overall survival; SR: Survival rate; NA: Not available.

Figure 2 Overall survival curves for the entire cohort comparing patients with 1, 2, 3, 4, 5, and ≥ 6 examined lymph nodes.

survival between the stage I and II groups (HR: 1.089, 95%CI: 0.793-1.497, *P* = 0.598 for stage II, referred to stage I), but the survival of patients with stage IIIA disease was significantly lower than that of patients with stage I disease (HR: 3.730, 95%CI: 2.635-5.280, *P* < 0.0001).

To identify the cutoff point for the optimal number of examined LNs, we compared the survival of the entire cohort with stage I, II and IIIA groups with X-tile software. The ranges for the significant dichotomization

LN numbers varied among the three stages. The largest survival difference was observed at 4 LNs for stage I disease (*P* = 0.004; Figure 3A), at 4 or 5 LNs for stage II disease (*P* < 0.001 for both; Figure 3B and C), and at 6 LNs for stage IIIA disease (*P* = 0.019; Figure 3D). For the entire cohort, the optimal number of examined LNs was 4, 5, or 6 (*P* < 0.001 for all; Figure 3E-G).

A stepwise Cox regression identified race and sex as significant prognostic factors for the entire cohort (Table 3); however, race was not a significant factor for patients with stage II and IIIA disease. Grade was a significant prognostic factor for patients with stage I, II, and IIIA disease but not for the entire cohort; and radiation therapy was a significant prognostic factor only for patients with stage IIIA disease.

DISCUSSION

GBC is associated with a high incidence of invasion through the layers of the gallbladder wall into adjacent structures and LNs. The influence of LN metastases on primary GBC is supported by one series of reports, which showed that the 5-year survival rate of T1N0 patients was 33% compared with a 3% survival rate in T1N1 patients[14]. As a consequence, several large-scale studies were conducted to examine the role of extended LN dissection to determine whether the removal of

Table 3 Multivariate analyses for overall survival in patients with lymph node-negative gallbladder carcinoma

Variable	Entire cohort n = 893 (100%)		Stage I [1] n = 228 (25.5%)		Stage II [1] n = 444 (49.7%)		Stage IIIA [1] n = 221 (24.7%)	
	HR (95%CI)	[a]P	HR (95%CI)	[b]P	HR (95%CI)	[c]P	HR (95%CI)	[d]P
Age, yr	1.001 (1.000-1.002)	0.504	1.001 (1.000-1.002)	0.934	1.001 (1.000-1.002)	0.449	1.001 (1.000-1.002)	0.057
Sex								
Male	1		1		1		1	
Female	0.684 (0.532-0.879)	0.003	0.317 (0.156-0.645)	0.002	0.553 (0.346-0.881)	0.013	0.494 (0.299-0.818)	0.006
Race								
White	1		1		1		1	
Black	1.475 (1.008-2.160)	0.046	4.593 (1.625-12.981)	0.004	1.645 (0.775-3.490)	0.195	2.245 (0.904-5.575)	0.082
Others	0.821 (0.551-1.224)	0.334	0.262 (1.975)	0.719	0.509 (0.245-1.056)	0.070	1.362 (0.609-3.047)	0.452
Grade1								
Well	1		1		1		1	
Moderate	0.840 (0.609-1.157)	0.286	0.418 (0.194-0.902)	0.026	1.568 (0.905-2.715)	0.109	0.297 (0.125-0.704)	0.006
Poor	1.291 (0.908-1.835)	0.155	1.339 (0.449-3.994)	0.601	2.412 (1.306-4.453)	0.005	0.629 (0.259-1.531)	0.307
Undifferentiated	2.101 (0.891-4.954)	0.090	/	/	4.059 (1.033-15.951)	0.045	1.434 (0.323-6.373)	0.636
Unknown	0.856 (0.503-1.455)	0.565	0.384 (0.134-1.106)	0.076	2.376 (0.857-6.583)	0.096	0.260 (0.068-1.003)	0.050
Radiation								
No	1		1		1		1	
Yes	0.727 (0.515-1.027)	0.071	1.055 (0.302-3.688)	0.933	0.781 (0.430-1.419)	0.417	0.390 (0.215-0.708)	0.002
No. of LNs examined	1.001 (1.000-1.002)	0.162	1.001 (1.000-1.002)	0.910	1.001 (1.000-1.002)	0.382	1.001 (1.000-1.002)	0.167
Stage1								
I	1		/	/	/	/	/	/
II	1.089 (0.793-1.497)	0.598	/	/	/	/	/	/
IIIA	3.730 (2.635-5.280)	0.000	/	/	/	/	/	/

[1]AJCC/TNM 8th edition. OS: Overall survival.

additional LN basins would influence the survival of GBC patients[9,15].

Early retrospective reports suggested improved survival for late-stage GBC patients treated with extended regional lymphadenectomy compared with standard regional lymphadenectomy[9]. However, the minimum clearance and/or number of LNs that should be examined have yet to be established. In the study, we evaluated the impact of the number of examined LNs on survival in N0 GBC. Using the SEER database, we discovered that the median number of examined LNs was two for the entire cohort, one for stage I, two for stage II, and two for stage IIIA disease. We used the smallest median value, 1 LN, as the basis for our categorization of the SEER patient cohort into six groups that reflected the extent of lymphadenectomy: 1 LN, 2 LNs, 3 LNs, 4 LNs, 5 LNs, and ≥ 6 LNs. There was a significant difference in survival among the six groups for the entire cohort and for the stage I and II groups but not for the stage IIIA group. With X-tile software, we found that the minimum number of LNs that should be examined was four for stage I, four or five for stage II, and six for stage IIIA disease. Therefore, for the entire cohort, the number of examined LNs should be at least six, which is exactly consistent with the AJCC guidelines.

The general phenomenon that the more LNs are examined, the better the survival for N0 disease, has some potential explanations. For example, the final LN count may be a proxy for surgeon experience and surgical technique and may be reflective of more thorough pathological assessment and identification of nodes from the surgical specimen[16]. It is also related to the

concept of stage migration, where inadequate removal of LNs may result in the misclassification of LN-positive patients as N0[17]. However, the removal of too many LNs may result in side effects such as lymphatic leakage. Our results were consistent with those of previous studies that investigated the relationship between LN count and survival in GBC patients and demonstrated that at least six LNs should be examined to improve survival after resection surgery[18]. Notably, except for LN dissection, nerve dissection may be required, especially for T3 or T4 disease[19].

In conclusion, the analysis suggests that examining higher numbers of LNs is associated with improved survival after resection surgery in N0 GBC. As recommended in the AJCC guidelines, at least six LNs should be examined for patients with N0 GBC.

ARTICLE HIGHLIGHTS

Research background

The American Joint Committee on Cancer (AJCC) tumor-node-metastasis staging system for gallbladder carcinoma (GBC) has been updated recently to the 8th edition. The N category is re-defined by the number of metastatic lymph nodes (LNs) instead of the location of the metastatic LNs, as defined in the 7th edition.

Research motivation

The new staging system for GBC has not been validated yet. Thus, we used Surveillance, Epidemiology, and End Result (SEER) database to evaluate its impact on clinical practice.

Research objectives

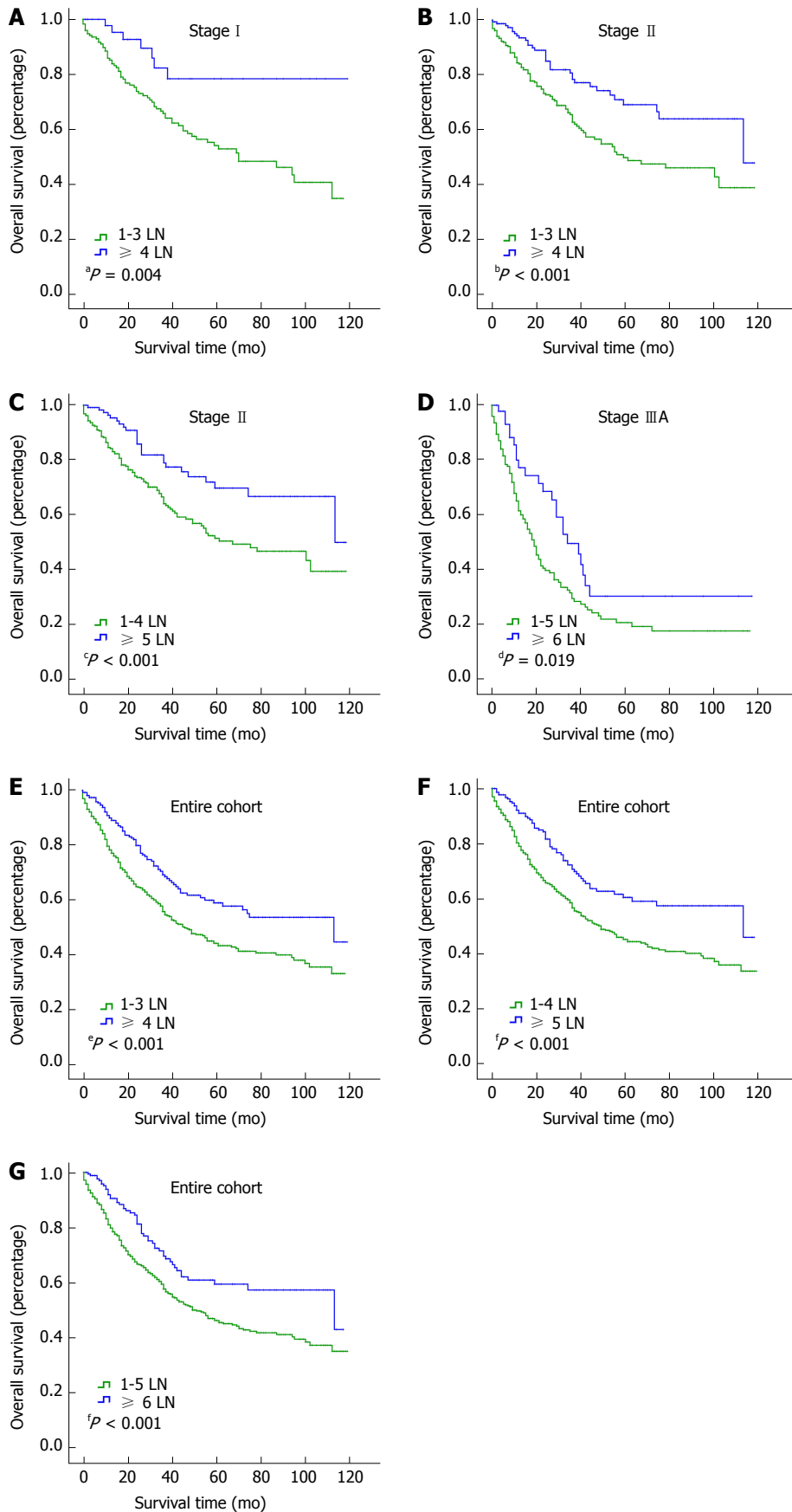The primary purpose of this study was to evaluate the impact of the number

Figure 3 Overall survival curves for the entire cohort and the stage Ⅰ, Ⅱ, and ⅢA groups and optimal dichotomization by the number of examined lymph nodes. A: Stage Ⅰ, LN = 4, P = 0.004; B: Stage Ⅱ, LN = 4, P ≤ 0.001; C: Stage Ⅱ, LN = 5, P ≤ 0.001; D: Stage ⅢA, LN = 6, P = 0.019; E: Entire cohort, LN = 4, P ≤ 0.001; F: Entire cohort, LN = 5, P ≤ 0.001; G: Entire cohort, LN = 6, P < 0.001. LN: Lymph node.

of examined LNs on the prognosis of N0 GBC. The secondary purpose was to verify the rationality of the guideline recommendation that at least six LNs should be harvested and evaluated.

Research methods

Patients were collected from the SEER database (2004-2013) and categorized by the number of LNs into six groups: 1 LN, 2 LNs, 3 LNs, 4 LNs, 5 LNs, and ≥ 6 LNs. Survival curves for overall survival were plotted with a Kaplan-Meier analysis. The log-rank test was used for univariate comparisons.

Research results

The survival for the 1 LN group was significantly lower than that of the stage Ⅰ and Ⅱ disease groups and for the entire cohort. By dichotomizing the number of LNs from one to six, we found that the minimum number of LNs that should be examined was four for stage Ⅰ, four or five for stage Ⅱ, and six for stage ⅢA disease. Thus, at least six LNs should be examined for the entire cohort, which was exactly consistent with the AJCC criteria.

Research conclusions

The examination of higher numbers of LNs is associated with improved survival after resection surgery for N0 GBC. As recommended in the guidelines, at least six LNs should be examined for patients with N0 GBC.

Research perspectives

The results validated the new recommendation in the AJCC guidelines, which can be applied widely in clinical practice.

REFERENCES

1   **Misra S**, Chaturvedi A, Misra NC, Sharma ID. Carcinoma of the gallbladder. *Lancet Oncol* 2003; **4**: 167-176 [PMID: 12623362 DOI: 10.1016/s1470-2045(03)01021-0]

2   **Randi G**, Franceschi S, La Vecchia C. Gallbladder cancer worldwide: geographical distribution and risk factors. *Int J Cancer* 2006; **118**: 1591-1602 [PMID: 16397865 DOI: 10.1002/ijc.21683]

3   **Meriggi F**. Gallbladder carcinoma surgical therapy. An overview. *J Gastrointestin Liver Dis* 2006; **15**: 333-335 [PMID: 17205143]

4   **Zhu AX**, Hong TS, Hezel AF, Kooby DA. Current management of gallbladder carcinoma. *Oncologist* 2010; **15**: 168-181 [PMID: 20147507 DOI: 10.1634/theoncologist.2009-0302]

5   **Reid KM**, Ramos-De la Medina A, Donohue JH. Diagnosis and surgical management of gallbladder cancer: a review. *J Gastrointest Surg* 2007; **11**: 671-681 [PMID: 17468929 DOI: 10.1007/s11605-006-0075-x]

6   **Chan SY**, Poon RT, Lo CM, Ng KK, Fan ST. Management of carcinoma of the gallbladder: a single-institution experience in 16 years. *J Surg Oncol* 2008; **97**: 156-164 [PMID: 18050290 DOI: 10.1002/jso.20885]

7   **Hong EK**, Kim KK, Lee JN, Lee WK, Chung M, Kim YS, Park YH. Surgical outcome and prognostic factors in patients with gallbladder carcinoma. *Korean J Hepatobiliary Pancreat Surg* 2014; **18**: 129-137 [PMID: 26155265 DOI: 10.14701/kjhbps.2014.18.4.129]

8   **Yamaguchi K**, Chijiiwa K, Saiki S, Nishihara K, Takashima M, Kawakami K, Tanaka M. Retrospective analysis of 70 operations for gallbladder carcinoma. *Br J Surg* 1997; **84**: 200-204 [PMID: 9052434 DOI: 10.1002/bjs.1800840217]

9   **Wang JD**, Liu YB, Quan ZW, Li SG, Wang XF, Shen J. Role of regional lymphadenectomy in different stage of gallbladder carcinoma. *Hepatogastroenterology* 2009; **56**: 593-596 [PMID: 19621661]

10 **Ott R**, Hauss J. Need and extension of lymph node dissection in gallbladder carcinoma. *Zentralbl Chir* 2006; **131**: 474-477 [PMID: 17206566 DOI: 10.1055/s-2006-955109]

11 **Sasaki R**, Takeda Y, Hoshikawa K, Takahashi M, Funato O, Nitta H, Murakami M, Kawamura H, Suto T, Yaegashi Y, Kanno S, Saito K. Long-term results of central inferior (S4a+S5) hepatic subsegmentectomy and pancreatoduodenectomy combined with extended lymphadenectomy for gallbladder carcinoma with subserous or mild liver invasion (pT2-3) and nodal involvement: a preliminary report. *Hepatogastroenterology* 2004; **51**: 215-218 [PMID: 15011867]

12 **Niu GC**, Shen CM, Cui W, Li Q. Surgical treatment of advanced gallbladder cancer. *Am J Clin Oncol* 2015; **38**: 5-10 [PMID: 25616200 DOI: 10.1097/COC.0b013e318287bb48]

13 **Tang Z**, Tian X and Wei M. Updates and interpretations of the 8th edition of AJCC cancer staging system for biliary tract carcinoma. *Chinese Journal of Practical Surgery* 2017; **37**: 248-254.

14 **Rückert JC**, Rückert RI, Gellert K, Hecker K, Müller JM. Surgery for carcinoma of the gallbladder. *Hepatogastroenterology* 1996; **43**: 527-533 [PMID: 8799389]

15 **Kondo S**, Nimura Y, Hayakawa N, Kamiya J, Nagino M, Kanai M, Uesaka K, Yuasa N, Sano T. [Value of paraaortic lymphadenectomy for gallbladder carcinoma]. *Nihon Geka Gakkai Zasshi* 1998; **99**: 728-732 [PMID: 9866839]

16 **Hellan M**, Sun CL, Artinyan A, Mojica-Manosa P, Bhatia S, Ellenhorn JD, Kim J. The impact of lymph node number on survival in patients with lymph node-negative pancreatic cancer. *Pancreas* 2008; **37**: 19-24 [PMID: 18580439 DOI: 10.1097/MPA.0b013e31816074c9]

17 **Feinstein AR**, Sosin DM, Wells CK. The Will Rogers phenomenon. Stage migration and new diagnostic techniques as a source of misleading statistics for survival in cancer. *N Engl J Med* 1985; **312**: 1604-1608 [PMID: 4000199 DOI: 10.1056/nejm198506203122504]

18 **Ito H**, Ito K, D'Angelica M, Gonen M, Klimstra D, Allen P, DeMatteo RP, Fong Y, Blumgart LH, Jarnagin WR. Accurate staging for gallbladder cancer: implications for surgical therapy and pathological assessment. *Ann Surg* 2011; **254**: 320-325 [PMID: 21617582 DOI: 10.1097/SLA.0b013e31822238d8]

19 **Ishihara S**, Miyakawa S, Takada T, Takasaki K, Nimura Y, Tanaka M, Miyazaki M, Nagakawa T, Kayahara M, Horiguchi A. Status of surgical treatment of biliary tract cancer. *Dig Surg* 2007; **24**: 131-136 [PMID: 17446708 DOI: 10.1159/000101901]

Observational Study

Upper gastrointestinal tract capsule endoscopy using a nurse-led protocol: First reported experience

Hey-Long Ching, Ailish Healy, Victoria Thurston, Melissa F Hale, Reena Sidhu, Mark E McAlindon

Hey-Long Ching, Ailish Healy, Victoria Thurston, Melissa F Hale, Reena Sidhu, Mark E McAlindon, Academic Department of Gastroenterology and Hepatology, Royal Hallamshire Hospital, Sheffield Teaching Hospitals NHS Trust, Sheffield S10 2JF, United Kingdom

ORCID number: Hey-Long Ching (0000-0003-3692-4187); Ailish Healy (0000-0002-9663-4846); Victoria Thurston (0000-0002-5243-1190); Melissa F Hale (0000-0002-2966-7568); Reena Sidhu (0000-0003-0820-2400); Mark E McAlindon (0000-0003-0985-3643).

Author contributions: McAlindon ME designed the study, assisted with interpretation of results and critically appraised the paper; Healy A and Thurston V led the data collection; Ching HL assisted with data collection, performed the data analysis, drafted the initial manuscript and is guarantor; Sidhu R assisted with interpretation of results and critically appraised the paper; Hale MF critically appraised the paper; all authors approved the final manuscript.

Institutional review board statement: This study was registered as service evaluation with the clinical effectiveness unit (CEU number 7073), Sheffield Teaching Hospitals NHS Foundation Trust, United Kingdom.

Informed consent statement: Capsule endoscopy was performed on patients who declined to undergo gastroscopy and all provided written informed consent for the capsule examination which was performed in all cases as part of routine clinical practice. The capsule examinations were not performed as part of a clinical research trial. In these patients who refused to have gastroscopy, the capsule endoscopy protocol was registered as a service evaluation with the department of clinical effectiveness unit (CEU number 7073, Sheffield Teaching Hospitals NHS Foundation Trust) and the evaluation is presented in this paper.

Conflict-of-interest statement: Professor McAlindon ME has acted as a consultant for Medtronic Ltd. All remaining authors have no conflict of interest to report.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read and prepared the manuscript in accordance with the STROBE statement.

Correspondence to: Hey-Long Ching, MBBS, BSc, MRCP, **Clinical Research Fellow,** Clinical Investigations Unit, P1, Royal Hallamshire Hospital, Sheffield Teaching Hospitals, Sheffield, S10 2JF, United Kingdom. hey-long.ching@sth.nhs.uk
Telephone: +44-1142-261180
Fax: +44-1142-712692

Abstract

AIM

To test the feasibility and performance of a novel upper gastrointestinal (GI) capsule endoscope using a nurse-led protocol.

METHODS

We conducted a prospective cohort analysis of patients who declined gastroscopy (oesophagogastroduodenoscopy, OGD) but who consented to upper GI capsule endoscopy. Patients swallowed the upper GI

capsule following ingestion of 1 liter of water (containing simethicone). A series of positional changes were used to exploit the effects of water flow and move the upper GI capsule from one gravity-dependent area to another using a nurse-led protocol. Capsule transit time, video reading time, mucosal visualisation, pathology detection and patient tolerance was evaluated.

RESULTS
Fifty patients were included in the study. The mean capsule transit times in the oesophagus and stomach were 28 s and 68 min respectively. Visualisation of the following major anatomical landmarks was achieved (graded 1-5: Poor to excellent): Oesophagus, 4.8 ($\pm$ 0.5); gastro-oesophageal junction (GOJ), 4.8 ($\pm$ 0.8); cardia, 4.8 ($\pm$ 0.8); fundus, 3.8 ($\pm$ 1.2); body, 4.5 ($\pm$ 1); antrum, 4.5 ($\pm$ 1); pylorus, 4.7 ($\pm$ 0.8); duodenal bulb, 4.7 ($\pm$ 0.7); second part of the duodenum (D2), 4.7 ($\pm$ 1). The upper GI capsule reached D2 in 64% of patients. The mean video reading time was 48 min with standard playback mode and 20 min using Quickview (P = 0.0001). No pathology was missed using Quickview. Procedural tolerance was excellent. No complications were seen with the upper GI capsule.

CONCLUSION
The upper GI capsule achieved excellent views of the upper GI tract. Future studies should compare the diagnostic accuracy between upper GI capsule and OGD.

Key words: Capsule endoscopy; Upper gastrointestinal; Gastroscopy; Oesophagus; Stomach

Core tip: The demand for diagnostic upper gastrointestinal (GI) endoscopy is high. Capsule endoscopy is well tolerated and is a first line small bowel investigative modality. Capsule endoscopy of the upper GI tract has previously been limited by technology and complexity of use. We demonstrate the feasibility of a nurse-led protocol using simple patient positional changes to move the novel upper GI capsule around a water-filled stomach. This technique provides excellent mucosal views in the oesophagus, stomach and (battery life allowing) duodenum and is well tolerated. The upper GI capsule might be a potential non-invasive, patient-friendly, alternative for diagnostic upper GI endoscopy.

INTRODUCTION
Capsule endoscopy is the method of choice to image the small bowel mucosa and is an accepted alternative to colonoscopy[1,2]. However, the short length of the oesophagus, the volume of the stomach and the convoluted shape of the gastroduodenum present challenges to its role as a non-invasive upper gastrointestinal examination technique: Transit can be rapid through a straight lumen[3] and visualisation may be limited to the dependent part of the stomach.

The PillCam® ESO2 capsule (Given Imaging Ltd., Yoqneam, Israel) has cameras at both ends and is capable of high image acquisition rates (18 frames per second) to maximise oesophageal imaging and a 30-min battery life. Meta-analyses have shown that it is an effective tool to detect Barrett's oesophagus, oesophageal varices and oesophagitis[4-6]. Three studies have also shown that it can be used to identify patients with suspected upper gastrointestinal bleeding who need gastroscopy[7-9]. In a comparative study in dyspeptic patients, Marelli *et al*[10] identified all major pathology detected by gastroscopy using an ESO2. These examinations were performed following a fast alone: better visualisation may be achieved after ingestion of simethicone and water to distend the stomach[11-13].

The upper gastrointestinal (UGI) capsule (Medtronic Ltd, Dublin, Ireland) represents the most recent technological advance in this field. Preserving dual-camera image capture, each with a 174° field of view, the UGI capsule captures as many as 35 frames per second for 10 min followed by 18 frames per second for a further 80 min. This study describes the first reported experience of UGI capsule endoscopy using a simple, nurse-led protocol comprising a sequence of patient positional changes following the ingestion of water and simethicone.

MATERIALS AND METHODS
Study population
We performed a prospective observational study at our tertiary hospital. Patients were offered UGI capsule endoscopy if they refused gastroscopy. All indications were considered. Those who had Crohn's disease were required to undergo a PillCam Patency capsule (Medtronic Ltd.) examination first.

Simple positional interchange technique
The UGI capsule endoscopy system includes an external portable data recorder. The recorder is connected to the patient by an array of leads on the chest and abdominal skin during the examination. This interface supports data export from the capsule to the memory drive of the data recorder. A small monitor in the recorder allows real-time viewing. When the procedure is complete, the data recorder is docked onto a workstation installed with Rapid 9® software (Medtronic Ltd.) and video images are exported for further analysis by the physician.

The simple positional interchange technique (SPIT) was performed by nursing staff on the Clinical Investigation Unit, Royal Hallamshire Hospital. Patients first drank one litre of water containing 80 mg simethicone. Immediately before swallowing the UGI capsule, 20 mg

Table 1 Upper gastrointestinal mucosal visualisation grading

Grade	Description
1	Poor view. More than 75% obscured by debris/bubbles/poor image clarity/illumination
2	Sub-optimal view. More than or equal to 50% obscured by debris/bubbles/poor image clarity/illumination
3	Reasonable view. Less than 50% obscured by debris/bubbles/poor image clarity/illumination
4	Good view. Less than 25% obscured by debris/bubbles/poor image clarity/illumination
5	Excellent. 100% complete view of the landmark

Views of each major landmark were graded; oesophagus, gastro-oesophageal junction; gastric cardia, fundus, body (anterior, posterior wall, greater and lesser curve), antrum, pylorus, and the first (D1) and second part of the duodenum (D2).

Figure 1 Schematic of the simple positional interchange technique. Coronal views are illustrated on the left and transverse views (with the cranial end closest to the reader) on the right. Capsule movement is achieved by exploiting the effects of water flow from one gravity dependent area to another with patient positional change. Once the UGI capsule enters the stomach, the examination bed is tilted 30° head down (depicted in blue) and patients lie supine (position 1), on their left lateral (position 2) and then prone (position 3). The bed is returned to the horizontal plane (depicted in green) and patients lie on their left lateral (position 4), supine (position 5) and then right lateral (position 6). The bed is finally adjusted to 30° head up (depicted in grey) and patients lie supine (position 7), on their left lateral (position 8) and then prone (position 9). UGI: Upper gastrointestinal.

of hyoscine butylbromide was given intramuscularly to reduce gastric peristalsis[14] and optimise gastric views. Patients were asked to swallow the UGI capsule in the right lateral position using an adaptation of the previously described simplified ingestion procedure (SIP)[15]. In brief, this entailed swallowing small sips of water (approximately 15mL) every 30 s until the UGI capsule entered the stomach. If patients were unable to swallow the capsule while lying in the horizontal plane, the head of the bed was incrementally elevated until swallowing was successful. If this failed, then patients swallowed the capsule sitting upright. The real-time views detected when the UGI capsule entered the stomach. Once the capsule entered the stomach, patients were asked to position themselves to face three planes (left/right lateral decubitus and supine/prone) at three angles (30° head down/up and horizontal) for 2 min per position (Figure 1). Additional positional changes and sips of water were used to improve views of the gastric mucosa as necessary. When complete gastric mucosal assessment was achieved patients were asked to sit

upright to assist passive capsule movement towards the pylorus. If the capsule had not reached the first part of the duodenum 60 min after ingestion then 10 mg of intramuscular metoclopramide was administered as per our standard protocol[11]. Patient tolerance in the form of procedural pain, discomfort and distress scores were recorded using previously validated visual analogue scales (VAS. 0: No symptom; 10: Intolerable symptom)[16,17].

Video interpretation and analysis
UGI capsule videos were reported by one of two co-authors (Sidhu R and McAlindon ME), each with experience of reading over 1000 small bowel capsule endoscopy videos. Rapid 9® software (Medtronic Ltd.) was used to review videos and has the capacity to playback recordings up to 100 frames per second in an accelerated reading mode. Analysis of videos included grading of mucosal visualisation (Table 1) using an adapted protocol[18]. Capsule transit time, video reading time, completion of examination to the second part of the duodenum (D2), pathology detection and procedural complications were recorded. The service evaluation was registered with the Clinical Effectiveness Unit (registration number 7073), Sheffield Teaching Hospitals NHS Foundation Trust (STH), United Kingdom.

SPSS V.22.0 (IBM) was used for statistical analysis. Continuous data was represented as mean ± SD: The student's t-test or one-way analysis of variance (ANOVA) was used for comparisons. Categorical data was represented as an absolute number and/or percentage: The χ^2 test or Fisher's exact probability test was used for comparisons. $P < 0.05$ (two-sided) was considered statistically significant.

RESULTS

Patient demographics
Fifty patients (40% male) with a mean age of 57 (± 15.7) years were included in the study protocol. Indications for investigation included dyspepsia (32%), iron deficiency anemia (14%), variceal screening (42%), suspected upper GI Crohn's disease (4%) and assessment of oesophageal ulcer healing (8%).

Performance characteristics
SPIT was achieved in 90% of patients: Five had difficulty lying prone. Complete examination to D2 was achieved
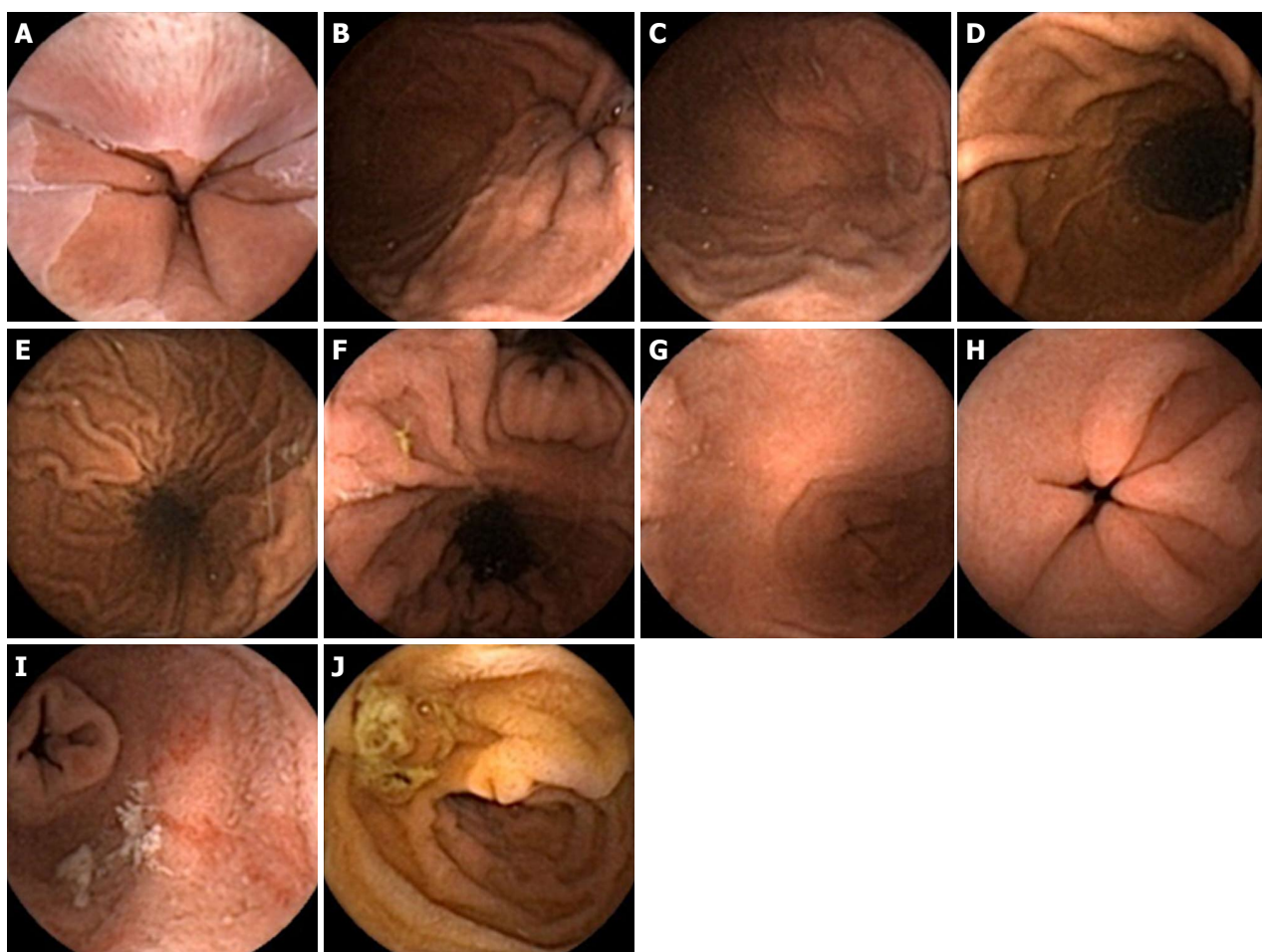
Figure 2 Normal views of the upper gastrointestinal tract seen with the upper gastrointestinal capsule. A: Gastroesophageal junction; B: Cardia; C: Fundus; D: Greater curvature; E: Lesser curvature; F: Incisura angularis; G: Antrum; H: Pylorus; I: First part of duodenum (retrograde view); J: Second part of duodenum (ampulla also seen).

in 64%. The mean ($\pm$ SD) time of capsule transit in the oesophagus, stomach and duodenum was 28 ($\pm$ 95) s, 68 ($\pm$ 25) min and 11 ($\pm$ 15) min respectively. Routine administration of hyoscine was abandoned after the first 33 patients because of concern that it might be delaying capsule entry into the duodenum. Analysis, however, failed to demonstrate any delaying effect of the drug on gastric transit: The mean gastric transit time with hyoscine butylbromide was 69 ($\pm$ 25) min and 66 ($\pm$ 26) min without ($P = 0.67$).

Mucosal visualisation and pathology detection
The mean reading time for capsule videos was 48 ($\pm$ 18) min with standard mode. All 50 studies were subsequently de-identified and re-read by one reader (MEM) in a randomised, blinded fashion using the Quick-view (Medtronic Ltd.) option in the pre-set mode (the software selecting 10% of the most relevant lesions for viewing by the reader) to examine the stomach (oesophagus and duodenum being read in standard mode with frame rate selected by the reader according to his usual practice): Reading time was significantly reduced to 20 ($\pm$ 5) min ($P = 0.0001$).

Visualisation of the upper GI tract was graded as follows: Oesophagus, 4.8 ($\pm$ 0.5); gastro-oesophageal junction (GOJ), 4.8 ($\pm$ 0.8); cardia, 4.8 ($\pm$ 0.8); fundus, 3.8 ($\pm$ 1.2); body, 4.5 ($\pm$ 1); antrum, 4.5 ($\pm$ 1); pylorus, 4.7 ($\pm$ 0.8); duodenal bulb (D1), 4.7 ($\pm$ 0.7); D2, 4.7 ($\pm$ 1) (Figure 2). Withdrawal of hyoscine administration did not affect any visualisation scores. The visualisation grade at the fundus was significantly lower when compared to all other areas of the upper GI tract ($P < 0.05$ for comparisons to the oesophagus, GOJ, cardia, body, D1 and D2) (Figure 3). The whole circumference of the Z-line was seen in 92.5% of cases. Inability to achieve prone positions during SPIT did not render lower overall gastric visualisation compared to complete SPIT; combined mean scores of cardia, fundus, body, antrum and pylorus visualisation were 4 ($\pm$ 1) *vs* 4.2 ($\pm$ 1.4), respectively ($P = 0.38$). Detected pathology included: oesophagitis ($n = 12$), Barrett's oesophagus ($n = 1$), hiatus hernias ($n = 7$), Cameron's ulcer ($n = 1$), gastric inlet patch ($n = 1$), oesophageal varices ($n = 8$), gastric varices ($n = 2$), portal hypertensive gastropathy ($n = 5$), gastritis ($n = 20$), benign gastric polyps ($n = 10$), gastric ulcers ($n = 2$), duodenitis ($n = 4$), duodenal polyp ($n = 1$), villous atrophy ($n = 1$) and angioectasia ($n = 7$) (Figure 4). No pathology was missed using

Figure 3 Suboptimal views in the fundus. A: Mucus; B: Bubbles; C: Insufficient distension.

Figure 4 Pathology detected by upper gastrointestinal capsule. A: Erosive esophagitis; B: Oesophageal varices; C: Barrett's oesophagus; D: Gastric ulcer; E: Gastric angioectasia; F: Portal hypertensive gastropathy; G: Benign cystic fundic gland polyps; H: Coeliac disease.

the Quickview reading software in the stomach when compared to standard mode.

Patient tolerance and safety

Mean procedural pain, discomfort and distress scores were: 0.4 (± 1), 0.4 (± 1) and 0.3 (± 0.9) respectively. No complications were seen. All patients were willing to undergo a repeat procedure if it was necessary.

DISCUSSION

UGI capsule endoscopy achieved oesophagogastric examination in all patients, although limited battery life precluded duodenal examination in a third. All studies using swallowed water for gastric distension, simethicone and the SPIT were performed by nursing staff according to protocol. Patients were able to comply with the SPIT in 90% of cases although difficulties with lying prone in the remainder did not affect outcome. SPIT provided excellent views of all areas of the oesophagus and stomach, both D1 and D2 were visualised clearly when the capsule traversed the pylorus within the 90-minute

time frame and pathology was identified throughout. The procedure was extremely well tolerated and no complications occurred.

Gastroscopy is performed in 1% of the United Kingdom population per annum[19]. In the United States, an increase in 50% of gastroscopy utilisation was estimated within the space of a decade between 2000 and 2010[20]. However, gastroscopy is an uncomfortable procedure[16,21,22] and the majority of findings do not significantly affect management[23]. This would suggest a role for a well-tolerated, non-invasive alternative that could select the minority of patients who need upper gastrointestinal biopsies or endoscopic therapy. Unlike the small and large bowel, which are long, relatively straight with constant lumina, the upper gastrointestinal tract comprises three quite different structures: the short, tubular, small diameter oesophagus and duodenum and the voluminous stomach, the gastroduodenum being convoluted in shape. Technologies to date have tried to address these challenges by developing capsules with cameras at both ends, maximising image capture rate and battery life and controlling capsule movement.

Although there is no equivalent data for the oesophagus, there is evidence that a double-ended pill camera is better than a single-ended one in terms of diagnostic yield in the small bowel[24,25]. Intuitively it seems likely that a single-ended capsule leading with the blind end is less likely to get complete views of the GOJ than one with cameras at both ends. Similarly, our experience is that a single ended device may miss proximal lesions in the duodenal bulb if transit through the bulb is rapid[26].

The Pillcam® ESO, capturing a total of fourteen frames (seven from each end) per second[27] was superseded by the ESO2[28], capturing a total of 18 frames per second. The 35 frames per second delivered by the UGI capsule would deliver almost 1000 oesophageal images in the average transit time of 28 s shown in our evaluation. This improvement is likely to have resulted in better oesophageal views: The entire GOJ was seen in only 50% of ESO2 studies[3] compared to 92.5% in this series. Whether or not this translates to better diagnostic yield in the oesophagus and the rest of the upper gastrointestinal tract needs to be confirmed.

We, and others, have demonstrated some degree of control with an external handheld magnet[11,29,30], which has shown promise in comparison with conventional gastroscopy[26,31]. Rey *et al*[32] visualised between 85%-93% of gastric landmarks in a controlled trial comparing gastroscopy with capsule endoscopy controlled using a large fixed external magnet developed by Olympus and Siemens. Both modalities identified 58% of pathologies and both missed lesions identified by the other. A similar system was found to have a sensitivity of only 62% in comparison to gastroscopy but only 21 of 189 patients recruited had focal pathology[33]. More recently, Liao *et al*[12] demonstrated that capsule endoscopy controlled by a robot magnet achieved 90% sensitivity (irrespective of size and location) in detecting focal lesions compared to gastroscopy in a large 350 patient multicenter study in Chinese patients with dyspepsia. Such techniques, however, require expertise and cost-effectiveness studies are needed. Therefore, the prospect of a simple, nurse-led, protocol driven UGI examination is attractive: cost and expertise required is mainly limited to the capsule and the interpretation of the videos.

The SPIT protocol is easy to follow in clinical practice. The patient is asked to rotate along their longitudinal axis almost 360° from the right lateral to prone position, a series of manoeuvres which are performed 30° head down, horizontal and 30° head up. This aims to achieve complete gastric imaging as was reported for capsule endoscopy using handheld external[34] and static robot magnets[35]. Qian *et al*[35] demonstrated the benefits of the left lateral, supine and right lateral positions for imaging the fundus, cardia and antropyloric regions respectively. Rahman *et al*[34] found that visualising incisura, antrum and pylorus was best achieved by using the handheld magnet to position the capsule opposite the gravity-dependent positions on the greater curve

and antrum in the supine patient. We have used the prone position to achieve the same capsule position and viewpoints. The combination of patient positional changes in Rahman's study achieved good to excellent views of all areas of the upper gastrointestinal tract. These previous studies were performed using single ended camera capsules: it is likely that greater coverage is obtained using a double-ended capsule providing a view of almost 360°. Studies comparing diagnostic yield of the two modalities are warranted. Five patients were unable to achieve the prone position but otherwise completed SPIT without obvious impact on landmark visualisation. Nonetheless, SPIT may not be feasible for all those with mobility restrictions.

Capsule reading was time consuming at 48 min and most of the viewing is repetitive gastric imaging making reading a tedious task. However, image recognition software continues to be developed which can exclude sequentially identical images, or select images which are different or identified as pathological, thereby reducing the size of the video to be viewed. The Quickview system is such a software and in its previous iteration in the Pillcam® SB2 (Given Imaging Ltd.) was shown to have a sensitivity of 92.3% in detecting small bowel pathology[36]. Perhaps such software may prove more useful in the large volume stomach in which the capsule images the same areas repeatedly, compared to the small bowel in which transit distally is more constant and subject to less repetitive imaging of the same region. No pathology was missed when Quickview was used to view the stomach. In this study, videos were re-read with Quickview in a randomised order and anonymised. Even so, they were re-read by MEM, one of the co-authors involved in the initial video interpretation using standard mode. Unbiased Quickview video interpretation by an independent reader, blinded to the findings at standard reading would provide more reliable comparison. Future larger comparative studies are needed to confirm the value Quickview in UGI capsule endoscopy.

The UGI capsule visualised the fundus less well. This is consistent with other studies using capsule endoscopy, even with external actuation techniques such as magnetic steering[18,30]. During gastroscopy, gas insufflation is used to inspect the proximal stomach, which is collapsed in the fasted state. While varying amounts of water have been used to distend the stomach during upper GI capsule endoscopy[10,11], we have previously shown that 1000 mL improves mucosal clarity and distension compared to 200 mL[11]. Some UGI videos were obscured by adherent mucus in the proximal stomach. The use of mucolytics such as N-acetylcysteine or pronase has been shown to be of benefit in improving mucosal visibility during gastroscopy[37-39], although this did not translate to the only capsule endoscopy study to date[40]. Routine use of hyoscine has been advocated to improve visualisation in OGD[14]. This did not appear to make a difference in our experience, although as with water- and gas- distension techniques and mucolytics, the potential benefits of these

agents should be investigated further.

A 64% complete examination to D2 was disappointing. Hyoscine may delay gastric emptying[41], but although this was not a study powered to investigate its effects, hyoscine did not appear to have an obvious effect on gastric transit in this small cohort. Meltzer *et al*[42] found that only one half of their ESO2 (30 min) examinations reached the duodenum. Using a modified version of the ESO2 (with a 90-min battery life) and pre-procedural intravenous erythromycin, Gralnek *et al*[7] achieved duodenal entry of the capsule in 97.8% of cases. Therefore the use of promotility agents might be considered, unless rendered redundant by further improvements in battery life.

The development of transnasal and single-fibre endoscopy as well as Cytosponge acknowledges the need for less-invasive technologies for upper gastrointestinal screening and surveillance[43]. In this feasibility study, anxiety, discomfort and pain scores associated with the UGI capsule and SPIT were excellent, consistent with previous studies of capsule endoscopy of the oesophagus[44,45], small bowel[16] and colon[46]. Furthermore, Gupta *et al*[47] found that adult subjects expressed a preference for capsule endoscopy compared to sedated endoscopy for Barrett's oesophagus screening, raising the possibility that compliance with investigation might be better if less-invasive techniques are offered.

There are limitations to this study and with the technologies. This is an observational cohort study that suggests that UGI capsule endoscopy is feasible, and when technological development allows more reliable duodenal imaging, randomised controlled trials of diagnostic yield compared to gastroscopy are needed. Cost effectiveness studies should consider the costs of the supporting systems and their maintenance (endoscopes, stack systems, monitors, computer software), disinfection, accessories and disposables (which includes the capsule), training requirements and the time taken to perform procedures (including interpreting images). Capsule endoscopy at present remains only diagnostic. The technology to biopsy lesions has been reported but remains in the experimental phase[48]. However, whilst most endoscopists have a low threshold for taking biopsies, the use of non-invasive tests for *Helicobacter pylori* might reduce this and our experience of investigating patients with dyspepsia is that biopsies only increased diagnostic yield by 2.4%[23].

Within the context of the limitations, this study shows that upper GI capsule endoscopy can be performed by nurses in a protocol-driven manner using the novel UGI capsule (Medtronic Ltd.). The SPIT, combined with gastric insufflation using water and simethicone appears to allow excellent visualisation of the whole stomach, albeit with slightly reduced visibility in the fundus. The oesophagus and gastro-oesophageal junction are well seen although further work is needed to allow more reliable visualisation of the duodenum. The procedure is extremely well tolerated by patients.

ARTICLE HIGHLIGHTS

Research background
Upper gastrointestinal (UGI) endoscopy (gastroscopy) is the method of choice to investigate dyspepsia, but is an uncomfortable test which carries the risk of intubation and sedation. Dyspepsia is a common symptom of which potential malignant lesions are an uncommon cause. Therefore a non-invasive alternative which might appropriately select those patients who require gastroscopy in order to obtain biopsy samples for histological analysis or for endotherapy is desirable. Capsule endoscopy is well tolerated and is a first line small bowel imaging tool, but lack of control of capsule movement limits visualisation to the dependent parts of the stomach only. Control can be achieved using external magnets, but this requires operator skill and magnetic devices which may be expensive. A simpler method would be to use swallowed water as a medium in which to move the capsule in the flow of water to different dependent parts of the stomach using patient positional change.

Research motivation
Several techniques using magnets to control capsule movement have been developed, but movement in water flow induced by patient positional change might offer an effective, simpler and less expensive alternative which has not been studied. An assessment of the areas of the upper gastrointestinal tract a capsule endoscope is capable of visualising is necessary in order to determine if such a technique might be feasible. Were this to be so, comparative trials with gastroscopy in identifying pathology would be warranted.

Research objectives
Our aims were to determine the visualisation quality of different upper gastrointestinal landmarks using a capsule endoscope moved around a water-filled stomach using a novel patient positional change technique, to assess procedural completion and patient tolerance of the procedure and time taken to read and report the videos.

Research methods
This was an observational study of a cohort of patients undergoing capsule endoscopy because they declined to undergo gastroscopy. Visualisation quality of different landmarks (oesophagus, gastro-oesophageal junction, cardia, fundus, body, antrum, pylorus, duodenal bulb and second part of duodenum) was scored (1-5: Poor-excellent) as was patient tolerance in terms of pain, discomfort and distress (0-10: No - intolerable). Video reading times in both standard and Quickview mode were compared.

Research results
Complete oesophagogastric examination was achieved with excellent views in all 50 patients. However, the battery-life for the UGI capsule expired before reaching D2 in 36%. Future adaptations are necessary to either promote earlier exiting of the capsule from the stomach into the duodenum (by positional change or prokinetics) or extend battery life. Reading time was lengthy, at 48 min. Using Quickview reduced this to 20 min and no pathology was missed. Further blinded comparative trials are needed to determine the reliability of Quickview in this setting. For patients, the procedure was extremely well tolerated and no complications were seen with the UGI capsule in this study.

Research conclusions
Our study demonstrates the feasibility of achieving excellent views of the oesophagus, stomach and duodenum (when seen) using a novel nurse-led protocol to move the upper gastrointestinal (GI) capsule through a series of patient positional changes. Future randomised control trials assessing diagnostic yield against gastroscopy will be needed to demonstrate reliability. However, the results we report suggest that this protocol may be a well-tolerated and less invasive alternative means to examining the upper GI tract endoscopically.

Research perspectives
These findings suggest that UGI capsule endoscopy is feasible, allows visualisation of all oesophagogastric landmarks and is extremely well tolerated by patients. Technological improvement, for example in battery life,

is likely to ensure more reliable imaging of the duodenum. If so, the simple positional interchange technique using the UGI capsule should be compared to gastroscopy in terms of diagnostic yield. Further studies to improve video reading time are needed.

REFERENCES

1   **Ladas SD**, Triantafyllou K, Spada C, Riccioni ME, Rey JF, Niv Y, Delvaux M, de Franchis R, Costamagna G; ESGE Clinical Guidelines Committee. European Society of Gastrointestinal Endoscopy (ESGE): recommendations (2009) on clinical use of video capsule endoscopy to investigate small-bowel, esophageal and colonic diseases. *Endoscopy* 2010; **42**: 220-227 [PMID: 20195992 DOI: 10.1055/s-0029-1243968]

2   **Pennazio M**, Spada C, Eliakim R, Keuchel M, May A, Mulder CJ, Rondonotti E, Adler SN, Albert J, Baltes P, Barbaro F, Cellier C, Charton JP, Delvaux M, Despott EJ, Domagk D, Klein A, McAlindon M, Rosa B, Rowse G, Sanders DS, Saurin JC, Sidhu R, Dumonceau JM, Hassan C, Gralnek IM. Small-bowel capsule endoscopy and device-assisted enteroscopy for diagnosis and treatment of small-bowel disorders: European Society of Gastrointestinal Endoscopy (ESGE) Clinical Guideline. *Endoscopy* 2015; **47**: 352-376 [PMID: 25826168 DOI: 10.1055/s-0034-1391855]

3   **Krok KL**, Wagennar RR, Kantsevoy SV, Thuluvath PJ. Esophageal capsule endoscopy is not the optimal technique to determine the need for primary prophylaxis in patients with cirrhosis. *Arch Med Sci* 2016; **12**: 365-371 [PMID: 27186182 DOI: 10.5114/aoms.2016.59263]

4   **Bhardwaj A**, Hollenbeak CS, Pooran N, Mathew A. A meta-analysis of the diagnostic accuracy of esophageal capsule endoscopy for Barrett's esophagus in patients with gastroesophageal reflux disease. *Am J Gastroenterol* 2009; **104**: 1533-1539 [PMID: 19491867 DOI: 10.1038/ajg.2009.86]

5   **Colli A**, Gana JC, Turner D, Yap J, Adams-Webber T, Ling SC, Casazza G. Capsule endoscopy for the diagnosis of oesophageal varices in people with chronic liver disease or portal vein thrombosis. *Cochrane Database Syst Rev* 2014; CD008760 [PMID: 25271409 DOI: 10.1002/14651858.CD008760.pub2]

6   **Lu Y**, Gao R, Liao Z, Hu LH, Li ZS. Meta-analysis of capsule endoscopy in patients diagnosed or suspected with esophageal varices. *World J Gastroenterol* 2009; **15**: 1254-1258 [PMID: 19291827 DOI: 10.3748/wjg.15.1254]

7   **Gralnek IM**, Ching JY, Maza I, Wu JC, Rainer TH, Israelit S, Klein A, Chan FK, Ephrath H, Eliakim R, Peled R, Sung JJ. Capsule endoscopy in acute upper gastrointestinal hemorrhage: a prospective cohort study. *Endoscopy* 2013; **45**: 12-19 [PMID: 23254402 DOI: 10.1055/s-0032-1325933]

8   **Meltzer AC**, Pinchbeck C, Burnett S, Buhumaid R, Shah P, Ding R, Fleischer DE, Gralnek IM. Emergency physicians accurately interpret video capsule endoscopy findings in suspected upper gastrointestinal hemorrhage: a video survey. *Acad Emerg Med* 2013; **20**: 711-715 [PMID: 23859585 DOI: 10.1111/acem.12165]

9   **Sung JJ**, Tang RS, Ching JY, Rainer TH, Lau JY. Use of capsule endoscopy in the emergency department as a triage of patients with GI bleeding. *Gastrointest Endosc* 2016; **84**: 907-913 [PMID: 27156655 DOI: 10.1016/j.gie.2016.04.043]

10   **Marelli L**, Jaboli FM, Jackson L, Palmer H, Erian G, Hamilton M, Epstein O. A pilot study comparing ESO-2 capsule endoscopy with conventional upper endoscopy for the assessment of uncomplicated heartburn and dyspepsia. *Frontline Gastroenterol* 2013; **4**: 96-101 [PMID: 28839708 DOI: 10.1136/flgastro-2012-100251]

11   **Hale MF**, Drew K, Sidhu R, McAlindon ME. Does magnetically assisted capsule endoscopy improve small bowel capsule endoscopy completion rate? A randomised controlled trial. *Endosc Int Open* 2016; **4**: E215-E221 [PMID: 26878053 DOI: 10.1055/s-0035-1569846]

12   **Liao Z**, Hou X, Lin-Hu EQ, Sheng JQ, Ge ZZ, Jiang B, Hou XH, Liu JY, Li Z, Huang QY, Zhao XJ, Li N, Gao YJ, Zhang Y, Zhou JQ, Wang XY, Liu J, Xie XP, Yang CM, Liu HL, Sun XT, Zou WB, Li ZS. Accuracy of Magnetically Controlled Capsule Endoscopy, Compared With Conventional Gastroscopy, in Detection of Gastric Diseases. *Clin Gastroenterol Hepatol* 2016; **14**: 1266-1273.e1 [PMID: 27211503 DOI: 10.1016/j.cgh.2016.05.013]

13   **Zou WB**, Hou XH, Xin L, Liu J, Bo LM, Yu GY, Liao Z, Li ZS. Magnetic-controlled capsule endoscopy vs. gastroscopy for gastric diseases: a two-center self-controlled comparative trial. *Endoscopy* 2015; **47**: 525-528 [PMID: 25590177 DOI: 10.1055/s-0034-1391123]

14   **Veitch AM**, Uedo N, Yao K, East JE. Optimizing early upper gastrointestinal cancer detection at endoscopy. *Nat Rev Gastroenterol Hepatol* 2015; **12**: 660-667 [PMID: 26260369 DOI: 10.1038/nrgastro.2015.128]

15   **Gralnek IM**, Rabinovitz R, Afik D, Eliakim R. A simplified ingestion procedure for esophageal capsule endoscopy: initial evaluation in healthy volunteers. *Endoscopy* 2006; **38**: 913-918 [PMID: 16981109 DOI: 10.1055/s-2006-944718]

16   **Irvine AJ**, Sanders DS, Hopper A, Kurien M, Sidhu R. How does tolerability of double balloon enteroscopy compare to other forms of endoscopy? *Frontline Gastroenterol* 2016; **7**: 41-46 [PMID: 28839833 DOI: 10.1136/flgastro-2014-100550]

17   **Elphick DA**, Donnelly MT, Smith KS, Riley SA. Factors associated with abdominal discomfort during colonoscopy: a prospective analysis. *Eur J Gastroenterol Hepatol* 2009; **21**: 1076-1082 [PMID: 19339891 DOI: 10.1097/MEG.0b013e32832357b3]

18   **Liao Z**, Duan XD, Xin L, Bo LM, Wang XH, Xiao GH, Hu LH, Zhuang SL, Li ZS. Feasibility and safety of magnetic-controlled capsule endoscopy system in examination of human stomach: a pilot study in healthy volunteers. *J Interv Gastroenterol* 2012; **2**: 155-160 [PMID: 23687601 DOI: 10.4161/jig.23751]

19   Provision of gastrointestinal endoscopy and related services for a district general hospital. Working Party of the Clinical Services Committee of the British Society of Gastroenterology. *Gut* 1991; **32**: 95-105 [PMID: 1991644 DOI: 10.1136/gut.32.1.95]

20   **Sonnenberg A**, Amorosi SL, Lacey MJ, Lieberman DA. Patterns of endoscopy in the United States: analysis of data from the Centers for Medicare and Medicaid Services and the National Endoscopic Database. *Gastrointest Endosc* 2008; **67**: 489-496 [PMID: 18179793 DOI: 10.1016/j.gie.2007.08.041]

21   **Brandt LJ**. Patients' attitudes and apprehensions about endoscopy: how to calm troubled waters. *Am J Gastroenterol* 2001; **96**: 280-284 [PMID: 11232665 DOI: 10.1111/j.1572-0241.2001.03508.x]

22   **Campo R**, Brullet E, Montserrat A, Calvet X, Moix J, Rué M, Roqué M, Donoso L, Bordas JM. Identification of factors that influence tolerance of upper gastrointestinal endoscopy. *Eur J Gastroenterol Hepatol* 1999; **11**: 201-204 [PMID: 10102233 DOI: 10.1097/00042737-199902000-00023]

23   **Ching HL**, Hale MF, Sidhu R, McAlindon ME. Reassessing the value of gastroscopy for the investigation of dyspepsia. *Frontline Gastroenterol* 2018; **9**: 62-66 [PMID: 29484162 DOI: 10.1136/flgastro-2017-100838]

24   **Triantafyllou K**, Papanikolaou IS, Papaxoinis K, Ladas SD. Two cameras detect more lesions in the small-bowel than one. *World J Gastroenterol* 2011; **17**: 1462-1467 [PMID: 21472105 DOI: 10.3748/wjg.v17.i11.1462]

25   **Remes-Troche JM**, Jiménez-García VA, García-Montes JM, Hergueta-Delgado P, Roesch-Dietlen F, Herrerías-Gutiérrez JM. Application of colon capsule endoscopy (CCE) to evaluate the whole gastrointestinal tract: a comparative study of single-camera and dual-camera analysis. *Clin Exp Gastroenterol* 2013; **6**: 185-192 [PMID: 24068872 DOI: 10.2147/CEG.S45215]

26   **Ching HL**, Hale MF, Sidhu R, Beg S, Ragunath K, McAlindon ME. Magnetically assisted capsule endoscopy (MACE) of the upper GI tract to select patients for endoscopy and reduce hospital admissions.: Presented at the BSG Annual Meeting 2017. Manchester, UK. BSG2017-942

27   **Koslowsky B**, Jacob H, Eliakim R, Adler SN. PillCam ESO in esophageal studies: improved diagnostic yield of 14 frames per second (fps) compared with 4 fps. *Endoscopy* 2006; **38**: 27-30 [PMID: 16429351 DOI: 10.1055/s-2005-921034]

28   **Laurain A**, de Leusse A, Gincul R, Vanbiervliet G, Bramli S, Heyries

L, Martane G, Amrani N, Serraj I, Saurin JC, Borentain P, Filoche B, Duburque C, Gaudric M, Sogni P, Dumortier J. Oesophageal capsule endoscopy versus oesophago-gastroduodenoscopy for the diagnosis of recurrent varices: a prospective multicentre study. *Dig Liver Dis* 2014; **46**: 535-540 [PMID: 24631032 DOI: 10.1016/j.dld.2014.02.002]

29 **Hale MF**, Rahman I, Drew K, Sidhu R, Riley SA, Patel P, McAlindon ME. Magnetically steerable gastric capsule endoscopy is equivalent to flexible endoscopy in the detection of markers in an excised porcine stomach model: results of a randomized trial. *Endoscopy* 2015; **47**: 650-653 [PMID: 25625696 DOI: 10.1055/s-0034-1391329]

30 **Rahman I**, Pioche M, Shim CS, Lee SP, Sung IK, Saurin JC, Patel P. Magnetic-assisted capsule endoscopy in the upper GI tract by using a novel navigation system (with video). *Gastrointest Endosc* 2016; **83**: 889-895.e1 [PMID: 26405045 DOI: 10.1016/j.gie.2015.09.015]

31 **Ching HL**, Hale MF, Campbell JA, Healy A, Thurston V, Sidhu R, et al. Magnetically steered capsule endoscopy (MSCE) of the upper and mid gut in recurrent and refractory iron deficiency anaemia. Presented at the BSG Annual Meeting 2017. Manchester, UK. BSG2017-1021

32 **Rey JF**, Ogata H, Hosoe N, Ohtsuka K, Ogata N, Ikeda K, Aihara H, Pangtay I, Hibi T, Kudo SE, Tajiri H. Blinded nonrandomized comparative study of gastric examination with a magnetically guided capsule endoscope and standard videoendoscope. *Gastrointest Endosc* 2012; **75**: 373-381 [PMID: 22154417 DOI: 10.1016/j.gie.2011.09.030]

33 **Denzer UW**, Rösch T, Hoytat B, Abdel-Hamid M, Hebuterne X, Vanbiervielt G, Filippi J, Ogata H, Hosoe N, Ohtsuka K, Ogata N, Ikeda K, Aihara H, Kudo SE, Tajiri H, Treszl A, Wegscheider K, Greff M, Rey JF. Magnetically guided capsule versus conventional gastroscopy for upper abdominal complaints: a prospective blinded study. *J Clin Gastroenterol* 2015; **49**: 101-107 [PMID: 24618504 DOI: 10.1097/MCG.0000000000000110]

34 **Rahman I**, Kay M, Bryant T, Pelitari S, Salter S, Dimitrov B, Patel P. Optimizing the performance of magnetic-assisted capsule endoscopy of the upper GI tract using multiplanar CT modelling. *Eur J Gastroenterol Hepatol* 2015; **27**: 460-466 [PMID: 25874522 DOI: 10.1097/MEG.0000000000000312]

35 **Qian Y**, Wu S, Wang Q, Wei L, Wu W, Wang L, Chu Y. Combination of Five Body Positions Can Effectively Improve the Rate of Gastric Mucosa's Complete Visualization by Applying Magnetic-Guided Capsule Endoscopy. *Gastroenterol Res Pract* 2016; **2016**: 6471945 [PMID: 28018426 DOI: 10.1155/2016/6471945]

36 **Koulaouzidis A**, Smirnidis A, Douglas S, Plevris JN. QuickView in small-bowel capsule endoscopy is useful in certain clinical settings, but QuickView with Blue Mode is of no additional benefit. *Eur J Gastroenterol Hepatol* 2012; **24**: 1099-1104 [PMID: 22668872 DOI: 10.1097/MEG.0b013e32835563ab]

37 **Basford PJ**, Brown J, Gadeke L, Fogg C, Haysom-Newport B, Ogollah R, Bhattacharyya R, Longcroft-Wheaton G, Thursby-Pelham F, Neale JR, Bhandari P. A randomized controlled trial of pre-procedure simethicone and N-acetylcysteine to improve mucosal visibility during gastroscopy - NICEVIS. *Endosc Int Open* 2016; **4**: E1197-E1202 [PMID: 27853746 DOI: 10.1055/s-0042-117631]

38 **Lee GJ**, Park SJ, Kim SJ, Kim HH, Park MI, Moon W. Effectiveness of Premedication with Pronase for Visualization of the Mucosa during Endoscopy: A Randomized, Controlled Trial. *Clin Endosc* 2012; **45**: 161-164 [PMID: 22866258 DOI: 10.5946/ce.2012.45.2.161]

39 **Kim GH**, Cho YK, Cha JM, Lee SY, Chung IK. Effect of pronase as mucolytic agent on imaging quality of magnifying endoscopy. *World J Gastroenterol* 2015; **21**: 2483-2489 [PMID: 25741158 DOI: 10.3748/wjg.v21.i8.2483]

40 **Zhu SG**, Qian YY, Tang XY, Zhu QQ, Zhou W, Du H, An W, Su XJ, Zhao AJ, Ching HL, McAlindon ME, Li ZS, Liao Z. Gastric preparation for magnetically controlled capsule endoscopy: A prospective, randomized single-blinded controlled trial. *Dig Liver Dis* 2018; **50**: 42-47 [PMID: 29110963 DOI: 10.1016/j.dld.2017.09.129]

41 **Stacher G**, Bergmann H, Havlik E, Schmierer G, Schneider C. Effects of oral cyclotropium bromide, hyoscine N-butylbromide and placebo on gastric emptying and antral motor activity in healthy man. *Gut* 1984; **25**: 485-490 [PMID: 6714792 DOI: 10.1136/gut.25.5.485]

42 **Meltzer AC**, Ali MA, Kresiberg RB, Patel G, Smith JP, Pines JM, Fleischer DE. Video capsule endoscopy in the emergency department: a prospective study of acute upper gastrointestinal hemorrhage. *Ann Emerg Med* 2013; **61**: 438-443.e1 [PMID: 23398660 DOI: 10.1016/j.annemergmed.2012.11.008]

43 **di Pietro M**, Chan D, Fitzgerald RC, Wang KK. Screening for Barrett's Esophagus. *Gastroenterology* 2015; **148**: 912-923 [PMID: 25701083 DOI: 10.1053/j.gastro.2015.02.012]

44 **Eliakim R**, Yassin K, Shlomi I, Suissa A, Eisen GM. A novel diagnostic tool for detecting oesophageal pathology: the PillCam oesophageal video capsule. *Aliment Pharmacol Ther* 2004; **20**: 1083-1089 [PMID: 15569110 DOI: 10.1111/j.1365-2036.2004.02206.x]

45 **Gralnek IM**, Adler SN, Yassin K, Koslowsky B, Metzger Y, Eliakim R. Detecting oesophageal disease with second-generation capsule endoscopy: initial evaluation of the PillCam ESO 2. *Endoscopy* 2008; **40**: 275-279 [PMID: 18389444 DOI: 10.1055/s-2007-995645]

46 **Ojidu H**, Palmer H, Lewandowski J, Hampton J, Blakeborough T, Epstein O, McAlindon ME. Patient tolerance and acceptance of different colonic imaging modalities: an observational cohort study. *Eur J Gastroenterol Hepatol* 2018; **30**: 520-525 [PMID: 29462029 DOI: 10.1097/MEG.0000000000001090]

47 **Gupta M**, Beebe TJ, Dunagan KT, Schleck CD, Zinsmeister AR, Talley NJ, Locke GR 3rd, Iyer PG. Screening for Barrett's esophagus: results from a population-based survey. *Dig Dis Sci* 2014; **59**: 1831-1850 [PMID: 24652109 DOI: 10.1007/s10620-014-3092-8]

48 **Koulaouzidis A**, Iakovidis DK, Karargyris A, Rondonotti E. Wireless endoscopy in 2020: Will it still be a capsule? *World J Gastroenterol* 2015; **21**: 5119-5130 [PMID: 25954085 DOI: 10.3748/wjg.v21.i17.5119]

Role of band ligation for secondary prophylaxis of variceal bleeding

Ioanna Aggeletopoulou, Christos Konstantakis, Spilios Manolakopoulos, Christos Triantos

Ioanna Aggeletopoulou, Christos Konstantakis, Christos Triantos, Department of Gastroenterology, University Hospital of Patras, Patras 26504, Greece

Spilios Manolakopoulos, 2nd Department of Internal Medicine, Hippokration General Hospital of Athens, Athens 11527, Greece

ORCID number: Ioanna Aggeletopoulou (0000-0003-4489-1485); Christos Konstantakis (0000-0001-5834-9182); Spilios Manolakopoulos (0000-0003-3130-7155); Christos Triantos (0000-0003-3094-8209).

Author contributions: Aggeletopoulou I and Konstantakis C were responsible for the literature review and analysis; Aggeletopoulou I, Manolakopoulos S and Triantos C were responsible for drafting the manuscript and interpretation of the data; Manolakopoulos S and Triantos C were responsible for revision of the manuscript for important intellectual content; all authors issued final approval for the version to be submitted.

Conflict-of-interest statement: Spilios Manolakopoulos has received research grants from Gilead Sciences, Regulus and Bristol-Myers Squibb and fees for lectures and advisory board from Gilead Sciences, Roche, Bristol-Myers Squibb, GSK, AbbVie and MSD; Christos Triantos has received fees as a speaker/advisory board member and research/travel grants from MSD, Roche, AbbVie, Janssen, Bristol-Myers Squibb, Bayer and Gilead Sciences.

PRISMA 2009 Checklist statement: The authors read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Manuscript source: Invited manuscript

Correspondence to: Christos Triantos, PhD, Assistant Professor in Internal Medicine and Gastroenterology, Department of Gastroenterology, University Hospital of Patras, D. Stamatopoulou 4, Rio, Patras 26504, Greece. chtriantos@hotmail.com
Telephone: +30-69-72894651
Fax: +30-26-10625382

Abstract

AIM
To summarize and critically examine the role of band ligation in secondary prophylaxis of variceal bleeding in patients with cirrhosis.

METHODS
A literature review was performed using the MEDLINE and PubMed databases. The search terms consisted of the words "endoscopic band ligation" OR "variceal band ligation" OR "ligation" AND "secondary prophylaxis" OR "secondary prevention" AND "variceal bleeding" OR "variceal hemorrhage" AND "liver cirrhosis". The data collected from relevant meta-analyses and from the most recent randomized studies that were not included in these meta-analyses were used to evaluate the role of endoscopic band ligation in an effort to demonstrate the most recent advances in the treatment of esophageal varices.

RESULTS
This study included 11 meta-analyses published from 2002 to 2017 and 10 randomized trials published from 2010 to 2017 that evaluated the efficacy of band ligation

in the secondary prophylaxis of variceal bleeding. Overall, the results proved that band ligation was superior to endoscopic sclerotherapy. Moreover, the use of β-blockers in combination with band ligation increased the treatment effectiveness, supporting the current recommendations for secondary prophylaxis of variceal bleeding. The use of transjugular intrahepatic portosystemic shunt was superior to combination therapy regarding rebleeding prophylaxis, with no difference in the survival rates; however, the results concerning the hepatic encephalopathy incidence were conflicting. Recent advances in the management of secondary prophylaxis of variceal bleeding have targeted a decrease in portal pressure based on the pathophysiological mechanisms of portal hypertension.

CONCLUSION
This review suggests that future research should be conducted to enhance current interventions and/or to develop innovative treatment options with improved clinical endpoints.

Key words: Band ligation; Variceal bleeding; Rebleeding; Liver cirrhosis; Endoscopic therapy; Variceal eradication; Secondary prophylaxis; Esophageal varices

Core tip: Variceal bleeding is a life-threatening complication of liver cirrhosis. The current guidelines recommend the use of band ligation together with β-blockers in the setting of secondary prophylaxis for variceal bleeding in patients with cirrhosis. This review summarizes data from meta-analyses and randomized trials to demonstrate the most recent advances in the management of variceal rebleeding. The current evidence suggests that the efficacy of band ligation is increased by adding β-blockers in accordance with the current guidelines. However, combination therapy does not procure a survival advantage. Innovative interventions and more effective novel strategies aiming to improve clinical outcomes should be developed.

INTRODUCTION

Approximately half of liver cirrhosis patients have developed gastroesophageal varices at diagnosis[1]. In the absence of proper treatment, approximately 30% of patients with varices will suffer a bleeding episode within the first 2 years following the cirrhosis diagnosis[2].

Variceal bleeding is considered one of the most severe complications of portal hypertension and constitutes a life-threatening condition for the cirrhosis patient. Patients surviving this first attack have an increased risk for rebleeding, especially during the first 6 weeks following the initial event. Overall, a second episode of variceal bleeding occurs in approximately 60% of this group of patients within 2 years[3,4]. The most common risk factors for variceal bleeding are the sizes of the varices[2,5,6], the severity of the liver disease[2] and the presence of red color signs on the variceal wall[2,7]. Patients with small varices have a low bleeding risk (approximately 5% per year), whereas patients with large varices have a higher bleeding rate of approximately 15% per year[1,8,9]. The 1-year bleeding probability in Child-Pugh class A cirrhosis patients with large varices and red signs is 24% compared with a 20% probability for Child-Pugh C patients with small varices and no red signs, indicating that variceal size constitutes the most useful predictor for variceal bleeding[2,6]. The aforementioned predictive factors have been combined in the North Italian Endoscopic Club index to classify patients according to the risk of a first variceal bleeding episode[2]. Variceal bleeding is associated with an increased hepatic venous pressure gradient (HVPG) (exceeding the threshold value of 12 mmHg). In contrast, a HVPG beneath 12 mmHg or a decrease in the HVPG gradient of more than 20% from the baseline level is related to a considerable reduction in the risk of variceal hemorrhage.

Patients who survive a first bleeding episode have a high risk of recurrence[8]. Therefore, these patients should receive appropriate treatment[10,11]. The primary aim of secondary prophylaxis is the prevention of further episodes of variceal hemorrhage and a reduction of associated mortality in cirrhosis patients. Available management options for secondary prophylaxis of variceal bleeding include pharmacotherapy, endoscopic treatment, transjugular intrahepatic portosystemic shunt (TIPS) and surgical shunting[8,12]. According to the Baveno VI guidelines and the practice guidance of the American Association for the Study of Liver Diseases (AASLD), the combination of non-selective β-blockers (propranolol or nadolol) and endoscopic band ligation constitutes the preferred treatment option for secondary prophylaxis in patients with liver cirrhosis[10,11]. Endoscopic band ligation should not be used alone unless the patient cannot tolerate β-blockers or there is a contraindication for non-selective β-blocker administration[10]. Patients who have not responded to the combination therapy should undergo covered TIPS insertion[10].

Systematic reviews and meta-analyses comparing these interventions have highlighted the differences in efficacy between the different modalities. The primary objective of this study is to summarize and critically review the existing data with a focus on the most updated randomized trials of the role of endoscopic band ligation in the secondary prophylaxis of variceal bleeding in liver cirrhosis patients.

MATERIALS AND METHODS

Search strategy

We conducted a review of the literature using the MEDLINE and PubMed databases. Data regarding the role of band ligation in secondary prophylaxis of variceal bleeding in liver cirrhosis patients were extracted from the relevant full articles. The search terms consisted of the words "endoscopic band ligation" OR "variceal band ligation" OR "ligation" AND "secondary prophylaxis" OR "secondary prevention" AND "variceal bleeding" OR "variceal hemorrhage" AND "liver cirrhosis".

Two reviewers (Aggeletopoulou I and Konstantakis C) independently reviewed all the titles and abstracts retrieved from the search after applying the inclusion criteria. A third reviewer (Triantos C) made the final decision in cases of disagreement. All manuscripts that compared endoscopic band ligation intervention *vs* other interventions were evaluated. Data collected from relevant meta-analyses and the most recent randomized studies not included in these meta-analyses were used to evaluate the role of endoscopic band ligation in an effort to demonstrate the most recent advances in the treatment of esophageal varices. All disagreements were resolved after full discussions within the research group.

Inclusion criteria

The inclusion criteria were as follows: (1) Full articles; (2) meta-analyses or systematic reviews comparing endoscopic band ligation *vs* other interventions (monotherapy or combination); (3) most recent randomized studies comparing endoscopic band ligation *vs* other interventions (monotherapy or combination) that were not included in the existing meta-analyses; (4) patients with liver cirrhosis; (5) studies containing the information of interest as subgroup analyses were included; and (6) Criteria 1 and 2 were applied in the setting of secondary prevention.

RESULTS

Meta-analyses

The meta-analyses that compared the effectiveness of endoscopic band ligation to that of other treatment options are presented in Table 1. Overall, 11 meta-analyses evaluated the efficacy of band ligation from 2002 to 2017[13-23]. Cheung *et al*[17] compared the efficacy of band ligation, pharmacotherapy [β-blockers alone or with isosorbide mononitrate (ISMN)] and their combination for the secondary prevention of variceal bleeding. The authors found no difference in the mortality and complication rates between the different treatment options and concluded that all treatment modalities were equally efficient for the prevention of rebleeding[17]. Similar results were found by Ding *et al*[13], who demonstrated no difference in rebleeding, mortality and complication rates between the band ligation and the β-blockers plus ISMN groups. Band ligation was compared with β-blockers plus ISMN in one additional meta-analysis; the results

showed no significant difference between band ligation and β-blockers with regard to all-cause mortality, bleeding-related mortality and the occurrence of adverse events[18]. However, a significant decrease in variceal bleeding was noted in patients who underwent band ligation compared to patients administered β-blockers that was attenuated when the analysis included only studies with adequate randomization and allocation concealment[18]. Thiele *et al*[19] assessed the effectiveness of band ligation with medical therapy compared with monotherapy (band ligation or medical therapy) and suggested that the combination treatment decreased the risk of rebleeding but did not influence the mortality rate compared with monotherapy. However, patients treated with combination therapy exhibited an increased trend towards the development of serious adverse events[19]. A subgroup analysis was performed in 2 meta-analyses to examine the efficacy of band ligation compared to band ligation plus pharmacotherapy; both meta-analyses agreed that combination therapy decreased the overall and variceal rebleeding rates[14,15]. Similar results reported by Ko *et al*[21] indicated that the combination therapy (β-blockers plus band ligation) was superior to pharmacotherapy alone for reduction of variceal rebleeding but not for overall rebleeding and mortality, which exhibited no differences between the two groups[21]. Lastly, another meta-analysis compared band ligation plus β-blockers to monotherapy (band ligation or β-blockers) after stratifying the patients according to their cirrhosis severity (Child-Pugh A *vs* B/C classes)[23]. The outcomes showed that the combination therapy was more effective in preventing rebleeding in the compensated patients but had no influence on the mortality rates[23]. In the decompensated patients, band ligation alone demonstrated an increased risk of rebleeding and mortality compared to combination therapy[23].

Nonsurgical therapeutic endoscopic approaches (endoscopic sclerotherapy and band ligation) for the control and prevention of bleeding episodes were compared by Dai *et al*[20], Karsan *et al*[22] and Singh *et al*[16]. Lower rebleeding, adverse event and mortality rates and higher variceal eradication were reported by Dai *et al*[20] in patients treated with band ligation compared to sclerotherapy, suggesting that endoscopic ligation should be the first-choice therapy. Furthermore, comparison of the combination of band ligation plus sclerotherapy with ligation alone failed to demonstrate significant differences in rebleeding prevention and mortality, and the former approach was associated with higher complication rates[16,22]. In contrast, a meta-analysis that evaluated the effectiveness of 12 prophylactic modalities for secondary prevention of variceal bleeding using multiple treatments indicated that band ligation combined with sclerotherapy could be used as a first-choice therapy[24]. Lastly, comparison of the efficacy of endoscopic procedures to that of pharmacotherapy showed that both methods were equally effective in terms of rebleeding prevention and all-cause mortality[25]. However, the combination of these methods was superior compared to endoscopic therapy

Table 1 Results from meta-analyses comparing band ligation with other interventions in terms of all-cause related rebleeding, variceal rebleeding, all-cause related mortality, bleeding related mortality and complication rates

Study (reference)	Publication year	Country	Method	Number of studies	Number of patients	All-cause related rebleeding RR or OR/CI//I^2	Variceal rebleeding RR or OR/CI //I^2	All-cause related mortality RR or OR/CI //I^2	Bleeding related mortality RR or OR/CI/ I^2	Complications RR or OR/CI/ I^2
Singh *et al*[16]	2002	United States	EBL *vs* EST + EBL	7	453	NR	1.12/ 0.69-1.81/ NR	NR	1.1/ 0.70-1.74/ NR	0.37/ 0.21-0.62/ NR
Karsan *et al*[22]	2005	United States	EBL *vs* EST + EBL	8	520	NR	1.05/ 0.67-1.64/ NS	0.99/ 0.68-1.44/ NS	NR	NR
[1]Gonzalez *et al*[15]	2008	Spain	[2]Combination therapy *vs* EBL	4	404	0.62/ 0.44-0.87/ 40%	NR	0.79/ 0.44-1.43/ 54%	NR	NR
Cheung *et al*[17]	2009	Canada	EBL *vs* PT	6	698	0.96/ 0.73-1.30/ 62%	NR/ NR/ 79%	1.20/ 0.92-1.57/ 0	NR	0.90/ 0.70-1.15/ 0
			EBL+PT *vs* EBL	4	404	0.57/ 0.31-1.08/ 60%	0.38/ 0.19-0.76/ 0	0.90/ 0.41-1.98/ 45%		3.4/ 1.4-8.2/ 74%
			EBL+PT *vs* PT	2	279	0.76/ 0.56-1.03/ 0	0.58/ 0.40-0.85/ 0	0.94/ 0.54-1.63/ 31%		NR
Ding *et al*[13]	2009	China	β-blockers + ISMN *vs* EBL	4	476	0.94/ 0.64-1.38 71.50%	NR	0.81/ 0.61-1.08/ 0	0.76/ 0.31-1.42/ 38.90%	1.26/ 0.93-1.70/ 42.70%
[1]Funakoshi *et al*[14]	2010	France	EBL *vs* EBL + β-blockers	3	252	3.16/ 1.76-5.34/ 0	NR	1.78/ 0.92-3.43/ 0	NR	NR
Li *et al*[18]	2011	China	EBL *vs* β-blockers + ISMN	6	687	0.95/ 0.65-1.40/ NR	0.89/ 0.53-1.49/ NR	1.25/ 1.01-1.55/ NR	1.16/ 0.68-1.97/ NR	NR
Thiele *et al*[19]	2012	Denmark	[3]EBL+PT *vs* monotherapy	9	955	0.68/ 0.54-0.85/ 1%	0.67/ 0.54-0.84/0	0.89/ 0.65-1.21/ 0	0.52/ 0.27-0.99/ NR	1.42/ 0.94-2.13/ 69%
Ko *et al*[21]	2012	South Korea	EBL + β-blockers *vs* β-blockers	4	409	0.78/ 0.58-1.04/ NR	0.60/ 0.41-0.88/ NR	1.21/ 0.88-1.65/ NR		
Dai *et al*[20]	2015	China	EBL *vs* EST	14	1236	0.68/ 0.57-0.81/ 9.00%	NR	0.95/ 0.77-1.17/ 32.80%	NR	0.28/ 0.13-0.58/ 86.50%
Albillos *et al*[23]	2017	Spain	EBL + β-blockers *vs* EBL	4	416	0.36/ 0.21-0.59/ NR	0.52/ 0.25-1.11/ NR	0.50/ 0.28-0.89/ NR	NR	NR
			EBL + β-blockers *vs* β-blockers	3	389	1.0/ 0.68-1.47/ NR	0.81/ 0.53-1.23/ NR	1.19/ 0.76-1.87/ NR		

[1]These results represent a subgroup analysis of the examined meta-analysis; [2]The term combination therapy includes endoscopic therapy combined with injection sclerotherapy or band ligation combined with drug therapy (β-blockers); [3]The term monotherapy includes endoscopic band ligation alone or medical therapy alone (β-blockers alone or combined with ISMN). RR: Risk ratio; OR: Odds ratio; CI: Confidence interval; I^2: Study heterogeneity; EBL: Endoscopic band ligation; EST: Endoscopic sclerotherapy; NR: Not reported; NS: Nonsignificant; PT: Pharmacotherapy; ISMN: Isosorbide mononitrate.

alone[25].

Randomized trials

The most recent randomized studies evaluating the role of band ligation in secondary prophylaxis (*vs* other interventions) that were not included in the existing meta-analyses were reviewed. Ten trials on secondary variceal bleeding prophylaxis in 770 patients with liver cirrhosis from 2010 to 2017 were included in this study (Table 2). The characteristics and clinical profiles of the

patients are summarized in Table 3.

Three trials compared the efficacy of band ligation *vs* endoscopic sclerotherapy[26-28], 3 trials compared band ligation *vs* pharmacotherapy[29-31], 2 trials compared band ligation *vs* TIPS[32,33], one trial compared band ligation *vs* cyanoacrylate injection[34] and one trial compared band ligation combined with sclerotherapy *vs* band ligation combined with microwave coagulation[35]. The results of these studies in terms of variceal obliteration, rebleeding and variceal recurrence are summarized in Table 4, and

Table 2 Characteristics of the included randomized trials

Study (reference)	Publication year	Country	Number of subjects
Monici *et al*[35]	2010	Brazil	70
Luz *et al*[26]	2011	Brazil	83
Santos *et al*[34]	2011	Brazil	38
Lo *et al*[31]	2013	Taiwan	118
Stanley *et al*[30]	2014	United Kingdom	64
Chen *et al*[28]	2016	China	96
Holster *et al*[32]	2016	Netherlands	72
Mansour *et al*[27]	2017	Egypt	120
Lv *et al*[33]	2017	China	49
Hanif *et al*[29]	2017	Pakistan	60

the results regarding mortality are summarized in Table 5.

Band ligation *vs* endoscopic sclerotherapy: Three studies evaluated the efficacy of band ligation *vs* endoscopic sclerotherapy alone[26] or in combination[27,28]. The comparison of band ligation *vs* endoscopic sclerotherapy showed no differences in bleeding control or in the early re-bleeding, complication and mortality rates[26]. Conflicting results emerged when band ligation was compared to sclerotherapy and band ligation[27,28]. Mansour *et al*[27] reported that sclerotherapy and band ligation were superior to band ligation for variceal obliteration, whereas Chen *et al*[28] showed that band ligation alone was more effective than the combination of ligation and sclerotherapy in terms of rebleeding. However, both studies demonstrated no differences in the adverse event rate and survival[27,28].

Band ligation *vs* pharmacotherapy: Stanley *et al*[30] assessed the efficacy of band ligation *vs* carvedilol and found no difference in the prevention of rebleeding. However, a trend towards an improved survival rate was observed in the patients who received carvedilol[30]. The effectiveness of band ligation plus propranolol *vs* propranolol alone was evaluated by Hanif *et al*[29], who suggested that the combination therapy was superior for secondary prophylaxis compared to the use of propranolol alone. Band ligation combined with proton pump inhibitors (PPIs) was compared to band ligation combined with vasoconstrictors; the results showed that adjuvant therapy with PPIs was similar to vasoconstrictors in relation to initial hemostasis and the very early rebleeding rate, but the combination treatment with PPIs demonstrated a lower rate of adverse events[31].

Band ligation *vs* TIPS: Band ligation plus β-blocker combination treatment was compared to TIPS in 2 trials. Both trials agreed that TIPS was superior to combination therapy for rebleeding prophylaxis; however, no difference was found in the survival rates[32,33].

Band ligation *vs* cyanoacrylate injection: One study evaluated the efficacy of band ligation compared to cyanoacrylate injection[34]. The results showed no significant difference between the two methods in terms of mortality, variceal obliteration and the adverse event rates but reported that patients treated with cyanoacrylate injection presented with more minor complications, earlier variceal recurrence and more bleeding episodes than the ligation group[34].

Band ligation plus sclerotherapy *vs* band ligation plus microwave coagulation: One study evaluated the rate of variceal recurrence in patients who received band ligation combined with either sequential microwave coagulation or endoscopic sclerotherapy in a cohort of Child-Pugh A and B patients[35]. The results showed that although the application of thermal therapy after ligation was safe and effective, no difference was found between the two methods in terms of variceal eradication, complications and variceal recurrence[35].

DISCUSSION

The aim of this review was to evaluate the effectiveness of endoscopic band ligation for secondary prophylaxis of esophageal variceal bleeding in liver cirrhosis patients. In this study, we incorporated data from meta-analyses that evaluated the efficacy of band ligation in comparison to (or in combination with) other interventions as well as the most recent data from randomized clinical trials that were not included in the aforementioned meta-analyses. We collected these data with the intention of identifying conflicting results from previous studies and obtaining precise estimates of treatment outcomes in terms of secondary prevention of variceal hemorrhage. Overall, current data favor the use of band ligation over endoscopic sclerotherapy. In addition, use of β-blockers combined with band ligation increases the treatment efficacy due to the reduced risk of rebleeding from the upper gastrointestinal system and esophageal varices. These findings are in agreement with the current clinical practice recommendations for secondary prophylaxis of variceal bleeding. Despite its proven benefits, the effect of combination therapy on survival remains uncertain. Therefore, further high-quality (and volume) studies and the development of novel treatment options are required.

Esophageal variceal bleeding constitutes a life-threatening complication of portal hypertension with a mortality rate of 12%-16% (depending on the analyzed cohort) and a high incidence of early rebleeding within the first 6 wk of the initial bleeding episode[36]. Endoscopic band ligation is a proven therapeutic option for achieving both initial hemostasis and preventing further bleeding episodes. The aim of band ligation is to eradicate varices through their "constriction" with rubber rings that are placed using a device attached to the endoscope tip called a "multiband ligator"[37]. The varices are sucked into the cap of the multiband ligator and then ligated through the release of a rubber band, which is responsible for the interruption of blood flow into the ligated varix[37]. Application of the bands initiates at

Table 3 Baseline characteristics of the patients included in the review

Study (reference)	Patients	Gender (M/F)	Age (range or ± SD)	CP class (A/B/C)	Cirrhosis etiology (agent %)
Monici *et al*[35]	EBL + EST: 36	25/11	47.8 (30-68)	28/8/0	Alcohol/virus/alcohol+virus/cryptogenic/autoimmune/PSC/PBC 12/13/3/5/2/1
	EBL + MC: 34	26/8	48.5 (22-71)	29/5/0	Alcohol/virus/alcohol+ virus/cryptogenic/autoimmune/PSC/PBC 8/11/3/9/1/2
Luz *et al*[26]	EBL: 44	NR	NR	2/22/20	Alcohol/virus/secondary biliary cirrhosis/cryptogenic/PBC 43.2/43.2/9.1/2.3/2.3
	EST: 39	NR	NR	3/21/15	Alcohol/virus/secondary biliary cirrhosis/cryptogenic/PBC 43.6/38.5/7.7/5.1/5.1
Santos *et al*[34]	EBL: 20	13/7	52 ± 12.6	0/4/16	Alcohol/HCV/alcohol+HCV/other 30/30/15/25
	CI: 18	14/4	51 ± 8.2	0/3/15	Alcohol/HCV/alcohol+HCV/other 39/33/6/22
Lo *et al*[31]	EBL+ vasoconstrictors: 60	49/11	52.5 ± 14.4	18/32/10	Alcohol/HBV/HCV/HBV+HCV/cryptogenic 40/22/30/3/5
	EBL+PPIs: 58	49/9	54.2 ± 9.7	15/24/19	Alcohol/HBV/HCV/HBV+HCV/cryptogenic 38/29/26/3/2/2
Stanley *et al*[30]	EBL: 31	21/10	49.6 ± 12.87	11/28/25	Alcohol/NAFLD/PBC/DICLD
	Carvedilol: 33	22/11	51.4 ± 10.8		91/5/3/2
Chen *et al*[28]	EBL: 48	32/16	56 ± 10	19/29/0	HBV/HCV/Alcohol/autoimmune/other 59/4/6/8/23
	EST: 48	31/17	54 ± 11	20/28/0	HBV/HCV/alcohol/autoimmune/other 75/0/2/10/13
Holster *et al*[32]	EBL+β-blockers: 35	23/12	54 (30-71)	13/18/4	Alcohol/HBV+HCV/alcohol + HBV+HCV/autoimmune liver+biliary disease/other 51/3/8/26/11
	TIPS: 37	18/19	56 (37-75)	13/19/5	Alcohol/HBV+HCV/alcohol + HBV+HCV/autoimmune liver+biliary disease/other 35/19/8/24/14
Mansour *et al*[27]	EBL: 60	34/26	NR	8/20/32	HCV/HBV/HCV+HBV 86.67/6.66/6.66
	EBL + EST: 60	44/16	NR	14/22/24	HCV/HBV/HCV+HBV 86.67/6.66/6.66
Lv *et al*[33]	EBL+propranolol: 25	16/8	46 (38-56)	10/14/1	HBV/HCV/alcohol/AH/HBV+AH/cryptogenic 86.67/13.3/0
	TIPS: 24	13/12	49 (46-62)	9/13/2	HBV/HCV/alcohol/AH/HBV+AH/cryptogenic 83/4/4/4/0/4
Hanif *et al*[29]	EBL+ propranolol: 30	25/5	56.30 ± 5.80	NR	NR
	Propranolol: 30	13/17	57.63 ± 5.98	NR	NR

CP: Child Pugh; EBL: Endoscopic band ligation; EST: Endoscopic sclerotherapy; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cirrhosis; MC: Microwave coagulation; NR: Not reported; HCV: Hepatitis C virus; HBV: Hepatitis B virus; CI: Cyanoacrylate injection; AH: Autoimmune hepatitis; NAFLD: Non-alcoholic fatty liver disease; PPIs: Proton pump inhibitors; DICLD: Drug-induced chronic liver disease; TIPS: Transjugular intrahepatic portosystemic shunt; NSBBs: Non-selective β-blockers.

the gastroesophageal junction and moves upwards in a helical manner for approximately 5-8 cm.

After initial control of bleeding, the band ligation sessions should be repeated at 1-wk to 4-wk intervals according to the practice guidelines of the AASLD[11] and at 1-wk to 8-wk intervals according to the American Society for Gastrointestinal Endoscopy guidelines[38] until the varices are eradicated. The complete eradication process typically requires 2 to 4 ligation sessions[39]. Variceal obliteration is achieved in approximately 90% of patients who undergo band ligation[40]. Once variceal obliteration has been achieved, a surveillance endoscopy is performed 3 mo to 6 mo after obliteration and every 6 to 12 mo thereafter to evaluate variceal recurrence[41]. Episodes of variceal recurrence after obliteration are common, with an incidence range of 20%-75% (within 1 year of therapy)[37].

The most recent consensus guidelines recommend the use of a combination of β-blockers and band ligation as the first-line therapy for the prevention of variceal rebleeding[10]. Non-selective adrenergic β-blockers, such as propranolol or nadolol, are preferred. The effect of non-selective adrenergic β-blockers relies on the reduction of portal pressure by decreasing the portal blood flow, because increased portal pressure is the driving force that enhances variceal growth and subsequent rupture, whereas band ligation only has a local effect[42]. The beneficial effect of combination treatment on variceal rebleeding was confirmed in 5 meta-analyses that assessed the efficacy of combined endoscopic and β-blocker therapy *vs* monotherapy in the prevention of variceal hemorrhage in cirrhosis patients[14,15,19,21,23]. However, the effect of combination therapy on survival was uncertain, because no significant

Table 4 Results of individual trials comparing band ligation with other interventions in terms of variceal obliteration, rebleeding and variceal recurrence

Study (reference)	Treatment	Mean sessions to obliterate	Rate of obliteration /time to obliterate (%)	Rebleeding rate (%)	Variceal recurrence rate
			Endoscopic band ligation *vs* endoscopic sclerotherapy		
Luz *et al*[26]	EBL	NR	75 at 5 d	25 at 5 d	NR
	EST		84.6 at 5 d	15.4 at 5 d	
Mansour *et al*[27]	EBL	3.43 ± 0.67	100 at 15.6 wk	16.70	26.7 at 3 mo
					10 at 6 mo
	EBL + EST	2.22 ± 0.92	100 at 8.64 wk	13.30	20 at 3 mo
					10 at 6 mo
Chen *et al*[28]	EBL	3 ± 0.5	25	14.60	NR
	EBL + EST	3 ± 0.6	16.30	35.40	
			Endoscopic band ligation *vs* β-blockers		
Stanley *et al*[30]	EBL	NR	65	35.50	NR
	Carvedilol		68	36.40	
			Endoscopic band ligation + β-blockers *vs* β-blockers		
Hanif *et al*[29]	EBL + propranolol	NR	NR	10	NR
	Propranolol			40	
			Endoscopic band ligation + PPIs *vs* endoscopic band ligation + vasoconstrictors		
Lo *et al*[31]	EBL + vasoconstrictors	NR	NR	1.7 at 6 d	NR
				8.3 at 6-42 d	
	EBL + PPIs			1.7 at 6 d	
				8.6 at 6-42 d	
			Endoscopic band ligation + β-blockers *vs* TIPS		
Holster *et al*[32]	EBL + β-blockers	NR	71 at 2 yr	26 at 2 yr	NR
	TIPS		73 at 2 yr	0 at 2 yr	
Lv *et al*[33]	EBL + propranolol	NR	NR	37 at 6 mo	NR
				45 at 12 mo	
				45 at 24 mo	
				52 at 30.4 mo	
	TIPS			5 at 6 mo	
				15 at 12 mo	
				20 at 24 mo	
				17 at 30.9 mo	
Santos *et al*[34]	EBL	3.17 ± 1.15	90 at 75.4 d	0	33 at 14.6 mo
	CI	3 ± 1.36	78 at 55.4 d	10	57 at 7.9 mo
			Endoscopic band ligation + endoscopic sclerotherapy *vs* endoscopic band ligation + microwave coagulation		
Monici *et al*[35]	EBL + EST	2.75 ± 1.92	97.30	8.30	27.7 at 9.5 mo
					19.5 at 12 mo
	EBL + MC	2.38 ± 1.63	97.10	0	17.6 at 9.16 mo
					17.5 at 12 mo

EBL: Endoscopic band ligation; EST: Endoscopic sclerotherapy; NR: Not reported; PPIs: Proton pump inhibitors; TIPS: Transjugular intrahepatic portosystemic shunt; CI: Cyanoacrylate injection; MC: Microwave coagulation.

difference was observed[14,15,19,21,23]. This result could be explained by a possible link between band ligation and the development of new or the exacerbation of previous complications, such as a ligation-related ulcer, portal hypertensive gastropathy, or the development of fundal varices[43-45]. The meta-analysis by Albillos *et al*[23] reported that the addition of band ligation with β-blockers resulted in a higher but not significant risk of mortality [incidence rate ratio (IRR) = 1.40; 95%CI: 0.87-2.27), all-source rebleeding (IRR = 1.36; 95%CI: 0.87-2.14) and variceal rebleeding (IRR = 1.24; 95%CI: 0.75-2.05) in patients with Child-Pugh class B/C, suggesting a potential deleterious effect of band ligation in this setting and highlighting the use of β-blockers as a key element of combination therapy[23]. The use of β-blockers enhances nonhemodynamic effects, such as a decrease in the drive of the sympathetic nervous system, and hemodynamic effects, such as a reduction in the splanchnic or gastroesophageal collateral blood flow and portal pressure[46,47]. Moreover, β-blockers may have a favorable effect on overall mortality, because they reduce the frequency of complications of cirrhosis, such as ascites, hepatorenal syndrome, portal hypertensive gastropathy[9,48] and spontaneous bacterial peritonitis[49-51]. A recent study described the "window hypothesis", which proposed that β-blockers had a beneficial impact on survival during the early phase of decompensated liver cirrhosis[46]. However, this benefit seems to diminish/disappear in well-compensated and end-stage cirrhosis patients[46,52]. Over the past few decades, variceal bleeding-related mortality has decreased. Conversely, deaths related other causes that are not associated with endoscopic treatment or pharmacotherapy, such as hepatocellular carcinoma, have demonstrated an increasing trend. Other studies, including a meta-analysis and four studies comparing

Table 5 Results of individual trials comparing band ligation with other interventions in terms of mortality

Study (reference)	Treatment	Mean hospitalization days (range or ± SD)	Mortality rate (%)	Follow up (range or ± SD)
		Endoscopic band ligation *vs* endoscopic sclerotherapy		
Luz *et al*[26]	EBL	NR	13.60	5 d
	EST		7.70	5 d
Mansour *et al*[27]	EBL	NR	No difference	6 mo
	EBL + EST			
		Endoscopic band ligation *vs* endoscopic band ligation + endoscopic sclerotherapy		
Chen *et al*[28]	EBL	NR	2.10	6 mo
	EBL + EST		6.30	
		Endoscopic band ligation *vs* β-blockers		
Stanley *et al*[30]	EBL	NR	51.60	26.3 mo
	Carvedilol		27.30	
		Endoscopic band ligation + β-blockers *vs* β-blockers		
Hanif *et al*[29]	EBL + propranolol	NR	NR	6 mo
	Propranolol			
		Endoscopic band ligation + PPIs *vs* endoscopic band ligation + vasoconstrictors		
Lo *et al*[31]	EBL + vasoconstrictors	9.4 ± 2.3	6.7 at 42 d	42 d
	EBL + PPIs	8.8 ± 3.8	5.2 at 42 d	
		Endoscopic band ligation + β-blockers *vs* TIPS		
Holster *et al*[32]	EBL + β-blockers	8.8 ± 5.4	20 at 2 yr	23.4 mo
	TIPS	12.4 ± 11.2	22 at 2 yr	
Lv *et al*[33]	EBL + propranolol	NR	12 at 6 mo	30.4 mo
			12 at 12 mo	
			16 at 24 mo	
			33 at 30.4 mo	
	TIPS		16 at 6 mo	30.9 mo
			17 at 12 mo	
			27 at 24 mo	
			33 at 30.9 mo	
		Endoscopic band ligation *vs* cyanoacrylate injection		
Santos *et al*[34]	EBL	NR	55	338 ± 189 d
	CI		56	
		Endoscopic band ligation + endoscopic sclerotherapy *vs* endoscopic band ligation + microwave coagulation		
Monici *et al*[35]	EBL + EST	NR	5.50	36.1 (15-53) mo
	EBL + MC		5.88	33.6 (14-54) mo

EBL: Endoscopic band ligation; EST: Endoscopic sclerotherapy; NR: Not reported; PPIs: Proton pump inhibitors; TIPS: Transjugular intrahepatic portosystemic shunt; CI: Cyanoacrylate injection; MC: Microwave coagulation.

band ligation *vs* combined ligation and β-blockers, found no significant differences in the rebleeding and mortality rates[17]. Several studies have compared the effectiveness of band ligation *vs* β-blockers with or without nitrates. Their results are compiled in 3 meta-analyses, which demonstrated comparable results for both the rebleeding and mortality rates[13,17,18].

Endoscopic sclerotherapy, which is another therapeutic intervention for variceal obliteration, has proven to be inferior to band ligation due to its higher complication rates and the number of sessions required for variceal obliteration[37,53]. However, endoscopic sclerotherapy achieves better results in cases of deeper paraesophageal varices, possibly because sclerotherapy induces fibrosis and eradication of perforating veins in contrast to band ligation, which does not affect collateral vessels in the deeper layers[54]. A randomized study that compared the early effects of endoscopic sclerotherapy *vs* band ligation on the HVPG values during acute bleeding episodes showed a sustained increase in the portal pressure levels after sclerotherapy that was followed by a higher rebleeding rate; in contrast, the HVPG values

in the ligation group returned to the baseline levels within 48 h[55]. A recent meta-analysis evaluated these two endoscopic approaches and concluded that band ligation was superior in terms of the rebleeding and mortality rates[20]. The combination of band ligation plus sclerotherapy was assessed by Singh *et al*[16] and Karsan *et al*[22], who found no advantage over ligation alone in the prevention of rebleeding and reduction of mortality.

Endoscopic band ligation, endoscopic sclerotherapy, drug therapy and TIPS constitute the nonsurgical therapeutic options for control of variceal bleeding and prevention of rebleeding episodes. Band ligation is considered the preferred initial approach, whereas TIPS is recommended in patients who fail endoscopic and pharmacological therapy or coagulation and those who are at high risk of treatment failure[10,11,56]. Portal vein thrombosis (PVT) is a frequent complication in patients with liver cirrhosis, with a prevalence rate ranging from 10% to 23%[57]. Acute variceal bleeding occurs in patients with PVT under certain circumstances. PVT is related to an increased risk of variceal bleeding and higher failure rates of primary and secondary prophylaxis of variceal

bleeding, resulting in higher mortality rates compared to those of cirrhosis patients without PVT. TIPS insertion has been well established as a safe and effective method for the secondary prophylaxis of variceal bleeding and recanalization of the portomesenteric system in patients with liver cirrhosis and PVT[58-62].

Recent randomized studies assessed the efficacy and safety of covered TIPS *vs* band ligation with β-blockers in patients with and without PVT[32,33]. Both studies suggested that TIPS implementation resulted in decreased variceal rebleeding rates but similar survival rates when compared to patients who received combination treatment[32,33]. In patients with PVT, TIPS insertion was also related to a higher rate of portal vein patency[33]. However, conflicting results were found regarding the incidence of hepatic encephalopathy. In patients with PVT, both groups demonstrated similar risks of hepatic encephalopathy[33]. In contrast, TIPS was associated with higher rates of early hepatic encephalopathy development in patients without PVT[32]. A meta-analysis showed a significant reduction in variceal rebleeding episodes and rebleeding-related mortality in patients undergoing TIPS *vs* endoscopic techniques; although TIPS increased the rate of post-treatment encephalopathy, the overall mortality rate remained the same for both groups[63]. Another meta-analysis that evaluated various interventions for secondary prophylaxis of variceal bleeding reported that TIPS, β-blockers combined with sclerotherapy and band ligation combined with sclerotherapy were superior to β-blockers alone in decreasing the rebleeding rates[24]. Moreover, TIPS was superior to β-blockers, band ligation, sclerotherapy, β-blockers combined with ISMN and β-blockers combined with sclerotherapy in terms of bleeding-related mortality[24]. These results were confirmed by a recent meta-analysis that evaluated the efficacy of TIPS compared to endoscopic treatment (band ligation, endoscopic sclerotherapy and cyanoacrylate injection) for the secondary prevention of variceal bleeding, the incidence of post-treatment hepatic encephalopathy and the survival of cirrhosis patients[64]. The results showed that the incidence of bleeding following TIPS was significantly lower than that in the endoscopic treatment group. Moreover, TIPS had a survival benefit in patients with Child-Pugh class C and those who underwent TIPS with a covered stent and did not increase the risk of hepatic encephalopathy. These results suggested that the use of covered TIPS was the preferred choice in patients with severe liver disease[64].

Other approaches that have been proposed to improve the outcome of band ligation, particularly variceal recurrence and rebleeding, include the following. Harras *et al*[65] proposed a combination of band ligation and argon plasma coagulation as an effective method to facilitate the rapid obliteration of varices accompanied by a low recurrence rate without obvious adverse events[65]. Another approach involves the injection of a monomeric liquid compound [cyanoacrylate (n-butyl-2-cyanoacrylate)], which is quickly polymerized when it comes into contact with the tissue surface and results in immediate eradication of the vessel[66]. Several randomized controlled studies have evaluated the efficacy of cyanoacrylate injection compared to other treatment modalities for esophageal varices[34,67,68]. Band ligation was compared with cyanoacrylate injection in two randomized studies, and the results showed no significant differences between the two groups in terms of variceal obliteration, mortality and major complications[34,67]. However, Santos *et al*[34] reported significantly more frequent minor complications, variceal recurrence and a clear trend towards an increase in bleeding episodes in the cyanoacrylate injection group than in the ligation group. Lastly, microwave coagulation, which is another thermal endoscopic treatment method, has been proposed in conjunction with band ligation for the treatment of esophageal varices[35]. Monici *et al*[35] evaluated the efficacy of band ligation plus microwave coagulation compared to band ligation plus endoscopic sclerotherapy and found that application of the microwave coagulation method was safe and gave similar results to the sclerotherapy group.

Recent advances in the management of secondary prophylaxis of variceal bleeding have emerged by targeting a decrease in portal pressure through the pathophysiological mechanisms of portal hypertension. First, the lipid-lowering agent simvastatin, which reduces the portal pressure and improves hepatocellular function, has been added to the standard treatment (β-blocker and band ligation) for variceal bleeding in cirrhosis patients. A recent placebo-controlled randomized trial showed that simvastatin administration was related to a significant amelioration of survival in Child-Pugh A and B patients[69]. However, no improvement was found in the rebleeding rates compared to those of patients who received the placebo[69]. Second, the use of alternative and more powerful β-blockers, which further reduce the HVPG compared to the effects of those used at present. The most recent guidelines recommend the use of propranolol or nadolol with or without ISMN for the prevention of variceal bleeding. However, reduction of HVPG is achieved in approximately 40% of patients, and the variceal bleeding risk is increased in hemodynamic non-responders. Studies have suggested that the use of carvedilol, which is a β-blocker with additional alpha-1 adrenoceptor inhibition properties, promotes a better hemodynamic response than propranolol or nadolol, prevents the progression of small esophageal varices and is more potent in reducing HVPG[70-73]. Lastly, portal pressure-guided therapy has been used to further improve the prevention of variceal rebleeding episodes. Villanueva *et al*[74] showed that the use of HVPG-guided therapy resulted in a significantly lower risk of rebleeding [hazard ratio (HR) = 0.53; 95%CI: 0.29-0.98], a decreased decompensation (HR = 0.68; 95%CI: 0.46-0.99), and mortality rate (HR = 0.59; 95%CI: 0.35-0.99) compared to the control group (combination of nadolol, nitrates and band ligation). Moreover, the hemodynamic responders in the HVPG-guided therapy group received

monotherapy with β-blockers, whereas the non-responders received combination treatment with β-blockers and band ligation. All patients in the control group received the combination treatment[74]. These results conclude that the addition of band ligation will not be beneficial for improving the outcomes if there is no hemodynamic response to β-blockers and set the stage for reevaluation of which patients should receive band ligation[75].

In conclusion, recently, management of variceal bleeding has markedly improved. These gains stem mainly from improvement of the overall strategy for secondary variceal prophylaxis of the cirrhosis population resulting from better understanding of the underlying mechanisms of the pathogenesis of portal hypertension, which guides the rationale behind each therapeutic intervention. In light of current evidence, endoscopic band ligation constitutes an effective treatment option for the prevention of recurrent variceal bleeding. However, the efficacy of band ligation is clearly increased by adding β-blocker therapy, and this combination is suggested as the first-line treatment for the prevention of rebleeding. Although the incidence of rebleeding is reduced by combined therapy in most studies, this option does not result in an overall survival advantage. However, other treatment modalities could also be considered in selected clinical scenarios. In the future, innovative endoscopic techniques and more effective treatment strategies or combinations of novel drugs should be developed with an aim of better clinical management of these patients.

ARTICLE HIGHLIGHTS

Research background
Variceal bleeding is considered one of the most severe complications of portal hypertension and constitutes a life-threatening condition for cirrhosis patients. Recurrent variceal bleeding occurs in approximately 60% of patients within 2 years, with a six-week mortality rate of approximately 12%-16%. Available treatments for the secondary prophylaxis of variceal bleeding include pharmacotherapy, endoscopic treatment, transjugular intrahepatic portosystemic shunt (TIPS) placement and surgical shunting. The most recent guidelines suggest that the combination of non-selective β-blockers (propranolol or nadolol) and endoscopic band ligation constitutes the preferred treatment option for prevention of rebleeding in liver cirrhosis patients. Endoscopic band ligation should not be used alone unless the patient cannot tolerate β-blockers or there is a contraindication for non-selective β-blocker administration. Covered TIPS insertion is recommended for patients who do not respond to combination treatment.

Research motivation
Systematic reviews and meta-analyses have compared these interventions and highlighted differences in the efficacy of the different modalities. However, conflicting data are present in the existing literature.

Research objectives
The authors aimed to summarize and critically examine existing data focusing on the most updated randomized trials of the role of endoscopic band ligation in the secondary prophylaxis of variceal bleeding in liver cirrhosis patients.

Research methods
A systematic search of the MEDLINE and PubMed databases was performed. All manuscripts comparing the endoscopic band ligation intervention *vs* other interventions were studied. Data from the relevant meta-analyses and the most recent randomized studies t not included in these meta-analyses were analyzed.

Research results
The results demonstrated that band ligation was more effective than endoscopic sclerotherapy. The use of β-blockers in combination with band ligation increased the treatment efficacy, supporting the current guidelines regarding secondary prevention of variceal bleeding. TIPS placement was superior to combination therapy in terms of rebleeding prophylaxis, with no difference in the survival rates. However, the data concerning the incidence of hepatic encephalopathy were conflicting.

Research conclusions
This review demonstrated the most recent advances in the role of endoscopic band ligation for the treatment of esophageal variceal rebleeding. Endoscopic band ligation constitutes an effective treatment option for the prevention of recurrent variceal bleeding. However, the efficacy of band ligation is clearly increased by the addition of β-blocker therapy. Other treatment modalities could also be considered in selected clinical scenarios.

Research perspectives
Innovative endoscopic techniques and more effective treatment strategies or combinations of novel drugs should be developed in the future, with an aim of better clinical management of these patients.

REFERENCES

1   **Haq I**, Tripathi D. Recent advances in the management of variceal bleeding. *Gastroenterol Rep* (Oxf) 2017; **5**: 113-126 [PMID: 28533909 DOI: 10.1093/gastro/gox007]

2   **North Italian Endoscopic Club for the Study and Treatment of Esophageal Varices**. Prediction of the first variceal hemorrhage in patients with cirrhosis of the liver and esophageal varices. A prospective multicenter study. *N Engl J Med* 1988; **319**: 983-989 [PMID: 3262200 DOI: 10.1056/NEJM198810133191505]

3   **D'Amico G**. Esophageal varices: from appearance to rupture; natural history and prognostic indicators. In: Groszmann RJ, Bosch J, editors. Portal Hypertension in the 21st Century. Dordrecht: Springer, 2004: 147-154 [DOI: 10.1007/978-94-007-1042-9_17]

4   **Poza Cordon J**, Froilan Torres C, Burgos García A, Gea Rodriguez F, Suárez de Parga JM. Endoscopic management of esophageal varices. *World J Gastrointest Endosc* 2012; **4**: 312-322 [PMID: 22816012 DOI: 10.4253/wjge.v4.i7.312]

5   **Polio J**, Groszmann RJ, Reuben A, Sterzel RB, Better OS. Portal hypertension ameliorates arterial hypertension in spontaneously hypertensive rats. *J Hepatol* 1989; **8**: 294-301 [PMID: 2732443 DOI: 10.1016/0168-8278(89)90026-3]

6   **Merkel C**, Zoli M, Siringo S, van Buuren H, Magalotti D, Angeli P, Sacerdoti D, Bolondi L, Gatta A. Prognostic indicators of risk for first variceal bleeding in cirrhosis: a multicenter study in 711 patients to validate and improve the North Italian Endoscopic Club (NIEC) index. *Am J Gastroenterol* 2000; **95**: 2915-2920 [PMID: 11051368 DOI: 10.1111/j.1572-0241.2000.03204.x]

7   **Groszmann RJ**, Bosch J, Grace ND, Conn HO, Garcia-Tsao G, Navasa M, Alberts J, Rodes J, Fischer R, Bermann M. Hemodynamic events in a prospective randomized trial of propranolol versus placebo in the prevention of a first variceal hemorrhage. *Gastroenterology* 1990; **99**: 1401-1407 [PMID: 2210246 DOI: 10.1016/0016-5085(90)91168-6]

8   **Bosch J**, García-Pagán JC. Prevention of variceal rebleeding. *Lancet* 2003; **361**: 952-954 [PMID: 12648985 DOI: 10.1016/S0140-6736(03)12778-X]

9   **Abraldes JG**, Tarantino I, Turnes J, Garcia-Pagan JC, Rodés J, Bosch J. Hemodynamic response to pharmacological treatment of portal hypertension and long-term prognosis of cirrhosis. *Hepatology* 2003; **37**: 902-908 [PMID: 12668985 DOI: 10.1053/jhep.2003.50133]

10   **de Franchis R**; Baveno VI Faculty. Expanding consensus in portal hypertension: Report of the Baveno VI Consensus Workshop: Stratifying risk and individualizing care for portal hypertension. *J Hepatol* 2015; **63**: 743-752 [PMID: 26047908 DOI: 10.1016/j.jhep.2015.05.022]

11   **Garcia-Tsao G**, Abraldes JG, Berzigotti A, Bosch J. Portal hypertensive bleeding in cirrhosis: Risk stratification, diagnosis, and management: 2016 practice guidance by the American Association for the study of liver diseases. *Hepatology* 2017; **65**: 310-335 [PMID: 27786365 DOI: 10.1002/hep.28906]

12   **Garcia-Tsao G**, Sanyal AJ, Grace ND, Carey W; Practice Guidelines Committee of the American Association for the Study of Liver Diseases; Practice Parameters Committee of the American College of Gastroenterology. Prevention and management of gastroesophageal varices and variceal hemorrhage in cirrhosis. *Hepatology* 2007; **46**: 922-938 [PMID: 17879356 DOI: 10.1002/hep.21907]

13   **Ding SH**, Liu J, Wang JP. Efficacy of beta-adrenergic blocker plus 5-isosorbide mononitrate and endoscopic band ligation for prophylaxis of esophageal variceal rebleeding: a meta-analysis. *World J Gastroenterol* 2009; **15**: 2151-2155 [PMID: 19418589 DOI: 10.3748/wjg.15.2151]

14   **Funakoshi N**, Ségalas-Largey F, Duny Y, Oberti F, Valats JC, Bismuth M, Daurès JP, Blanc P. Benefit of combination β-blocker and endoscopic treatment to prevent variceal rebleeding: a meta-analysis. *World J Gastroenterol* 2010; **16**: 5982-5992 [PMID: 21157975]

15   **Gonzalez R**, Zamora J, Gomez-Camarero J, Molinero LM, Bañares R, Albillos A. Meta-analysis: Combination endoscopic and drug therapy to prevent variceal rebleeding in cirrhosis. *Ann Intern Med* 2008; **149**: 109-122 [PMID: 18626050 DOI: 10.7326/0003-4819-149-2-200807150-00007]

16   **Singh P**, Pooran N, Indaram A, Bank S. Combined ligation and sclerotherapy versus ligation alone for secondary prophylaxis of esophageal variceal bleeding: a meta-analysis. *Am J Gastroenterol* 2002; **97**: 623-629 [PMID: 11922557 DOI: 10.1111/j.1572-0241.2002.05540.x]

17   **Cheung J**, Zeman M, van Zanten SV, Tandon P. Systematic review: secondary prevention with band ligation, pharmacotherapy or combination therapy after bleeding from oesophageal varices. *Aliment Pharmacol Ther* 2009; **30**: 577-588 [PMID: 19558563 DOI: 10.1111/j.1365-2036.2009.04075.x]

18   **Li L**, Yu C, Li Y. Endoscopic band ligation versus pharmacological therapy for variceal bleeding in cirrhosis: a meta-analysis. *Can J Gastroenterol* 2011; **25**: 147-155 [PMID: 21499579 DOI: 10.1155/2011/346705]

19   **Thiele M**, Krag A, Rohde U, Gluud LL. Meta-analysis: banding ligation and medical interventions for the prevention of rebleeding from oesophageal varices. *Aliment Pharmacol Ther* 2012; **35**: 1155-1165 [PMID: 22449261 DOI: 10.1111/j.1365-2036.2012.05074.x]

20   **Dai C**, Liu WX, Jiang M, Sun MJ. Endoscopic variceal ligation compared with endoscopic injection sclerotherapy for treatment of esophageal variceal hemorrhage: a meta-analysis. *World J Gastroenterol* 2015; **21**: 2534-2541 [PMID: 25741164 DOI: 10.3748/wjg.v21.i8.2534]

21   **Ko SY**, Kim JH, Choe WH, Kwon SY, Lee CH. Pharmacotherapy alone vs endoscopic variceal ligation combination for secondary prevention of oesophageal variceal bleeding: meta-analysis. *Liver Int* 2012; **32**: 867-869 [PMID: 22133043 DOI: 10.1111/j.1478-3231.2011.02681.x]

22   **Karsan HA**, Morton SC, Shekelle PG, Spiegel BM, Suttorp MJ, Edelstein MA, Gralnek IM. Combination endoscopic band ligation and sclerotherapy compared with endoscopic band ligation alone for the secondary prophylaxis of esophageal variceal hemorrhage: a meta-analysis. *Dig Dis Sci* 2005; **50**: 399-406 [PMID: 15745108 DOI: 10.1007/s10620-005-1618-9]

23   **Albillos A**, Zamora J, Martínez J, Arroyo D, Ahmad I, De-la-Peña J, Garcia-Pagán JC, Lo GH, Sarin S, Sharma B, Abraldes JG, Bosch J, Garcia-Tsao G; Baveno Cooperation. Stratifying risk in the prevention of recurrent variceal hemorrhage: Results of an individual

patient meta-analysis. *Hepatology* 2017; **66**: 1219-1231 [PMID: 28543862 DOI: 10.1002/hep.29267]

24   **Shi KQ**, Liu WY, Pan ZZ, Ling XF, Chen SL, Chen YP, Fan YC, Zheng MH. Secondary prophylaxis of variceal bleeding for cirrhotic patients: a multiple-treatments meta-analysis. *Eur J Clin Invest* 2013; **43**: 844-854 [PMID: 23725530 DOI: 10.1111/eci.12115]

25   **Ravipati M**, Katragadda S, Swaminathan PD, Molnar J, Zarling E. Pharmacotherapy plus endoscopic intervention is more effective than pharmacotherapy or endoscopy alone in the secondary prevention of esophageal variceal bleeding: a meta-analysis of randomized, controlled trials. *Gastrointest Endosc* 2009; **70**: 658-664.e5 [PMID: 19643407 DOI: 10.1016/j.gie.2009.02.029]

26   **Luz GO**, Maluf-Filho F, Matuguma SE, Hondo FY, Ide E, Melo JM, Cheng S, Sakai P. Comparison between endoscopic sclerotherapy and band ligation for hemostasis of acute variceal bleeding. *World J Gastrointest Endosc* 2011; **3**: 95-100 [PMID: 21772940 DOI: 10.4253/wjge.v3.i5.95]

27   **Mansour L**, El-Kalla F, El-Bassat H, Abd-Elsalam S, El-Bedewy M, Kobtan A, Badawi R, Elhendawy M. Randomized controlled trial of scleroligation versus band ligation alone for eradication of gastroesophageal varices. *Gastrointest Endosc* 2017; **86**: 307-315 [PMID: 28082116 DOI: 10.1016/j.gie.2016.12.026]

28   **Chen J**, Zeng XQ, Ma LL, Li B, Tseng YJ, Lian JJ, Gao H, Wang J, Luo TC, Chen SY. Randomized controlled trial comparing endoscopic ligation with or without sclerotherapy for secondary prophylaxis of variceal bleeding. *Eur J Gastroenterol Hepatol* 2016; **28**: 95-100 [PMID: 26517621 DOI: 10.1097/MEG.0000000000000499]

29   **Hanif M**, Hussain A, Aamer M, Adrees M, Shakoor S. Comparison between Combination of Band ligation and Propranolol with Propranolol alone in Secondary Prophylaxis of Variceal bleed. *APMC* 2017; **11**: 141-145

30   **Stanley AJ**, Dickson S, Hayes PC, Forrest EH, Mills PR, Tripathi D, Leithead JA, MacBeth K, Smith L, Gaya DR, Suzuki H, Young D. Multicentre randomised controlled study comparing carvedilol with variceal band ligation in the prevention of variceal rebleeding. *J Hepatol* 2014; **61**: 1014-1019 [PMID: 24953021 DOI: 10.1016/j.jhep.2014.06.015]

31   **Lo GH**, Perng DS, Chang CY, Tai CM, Wang HM, Lin HC. Controlled trial of ligation plus vasoconstrictor versus proton pump inhibitor in the control of acute esophageal variceal bleeding. *J Gastroenterol Hepatol* 2013; **28**: 684-689 [PMID: 23278466 DOI: 10.1111/jgh.12107]

32   **Holster IL**, Tjwa ET, Moelker A, Wils A, Hansen BE, Vermeijden JR, Scholten P, van Hoek B, Nicolai JJ, Kuipers EJ, Pattynama PM, van Buuren HR. Covered transjugular intrahepatic portosystemic shunt versus endoscopic therapy + β-blocker for prevention of variceal rebleeding. *Hepatology* 2016; **63**: 581-589 [PMID: 26517576 DOI: 10.1002/hep.28318]

33   **Lv Y**, Qi X, He C, Wang Z, Yin Z, Niu J, Guo W, Bai W, Zhang H, Xie H, Yao L, Wang J, Li T, Wang Q, Chen H, Liu H, Wang E, Xia D, Luo B, Li X, Yuan J, Han N, Zhu Y, Xia J, Cai H, Yang Z, Wu K, Fan D, Han G; PVT-TIPS Study Group. Covered TIPS versus endoscopic band ligation plus propranolol for the prevention of variceal rebleeding in cirrhotic patients with portal vein thrombosis: a randomised controlled trial. *Gut* 2017; Epub ahead of print [PMID: 28970291 DOI: 10.1136/gutjnl-2017-314634]

34   **Santos MM**, Tolentino LH, Rodrigues RA, Nakao FS, Rohr MR, de Paulo GA, Kondo M, Ferrari AP, Libera ED. Endoscopic treatment of esophageal varices in advanced liver disease patients: band ligation versus cyanoacrylate injection. *Eur J Gastroenterol Hepatol* 2011; **23**: 60-65 [PMID: 21084988 DOI: 10.1097/MEG.0b013e3283415986]

35   **Monici LT**, Meirelles-Santos JO, Soares EC, Mesquita MA, Zeitune JM, Montes CG, Almeida JR, Yamanaka A, Magna LA. Microwave coagulation versus sclerotherapy after band ligation to prevent recurrence of high risk of bleeding esophageal varices in Child-Pugh's A and B patients. *J Gastroenterol* 2010; **45**: 204-210 [PMID: 19802519 DOI: 10.1007/s00535-009-0134-7]

36   **Garcia-Tsao G**, Bosch J. Management of varices and variceal

hemorrhage in cirrhosis. *N Engl J Med* 2010; **362**: 823-832 [PMID: 20200386 DOI: 10.1056/NEJMra0901512]

37   Garcia-Pagán JC, Bosch J. Endoscopic band ligation in the treatment of portal hypertension. *Nat Clin Pract Gastroenterol Hepatol* 2005; **2**: 526-535 [PMID: 16355158 DOI: 10.1038/ncpgasthep0323]

38   Hwang JH, Shergill AK, Acosta RD, Chandrasekhara V, Chathadi KV, Decker GA, Early DS, Evans JA, Fanelli RD, Fisher DA, Foley KQ, Fonkalsrud L, Jue T, Khashab MA, Lightdale JR, Muthusamy VR, Pasha SF, Saltzman JR, Sharaf R, Cash BD; American Society for Gastrointestinal Endoscopy. The role of endoscopy in the management of variceal hemorrhage. *Gastrointest Endosc* 2014; **80**: 221-227 [PMID: 25034836 DOI: 10.1016/j.gie.2013.07.023]

39   Saeed ZA, Stiegmann GV, Ramirez FC, Reveille RM, Goff JS, Hepps KS, Cole RA. Endoscopic variceal ligation is superior to combined ligation and sclerotherapy for esophageal varices: a multicenter prospective randomized trial. *Hepatology* 1997; **25**: 71-74 [PMID: 8985267 DOI: 10.1002/hep.510250113]

40   Khuroo MS, Khuroo NS, Farahat KL, Khuroo YS, Sofi AA, Dahab ST. Meta-analysis: endoscopic variceal ligation for primary prophylaxis of oesophageal variceal bleeding. *Aliment Pharmacol Ther* 2005; **21**: 347-361 [PMID: 15709985 DOI: 10.1111/j.1365-2036.2005.02346.x]

41   Baron TH, Wong Kee Song LM. Endoscopic variceal band ligation. *Am J Gastroenterol* 2009; **104**: 1083-1085 [PMID: 19417747 DOI: 10.1038/ajg.2008.17]

42   Albillos A, Tejedor M. Secondary prophylaxis for esophageal variceal bleeding. *Clin Liver Dis* 2014; **18**: 359-370 [PMID: 24679500 DOI: 10.1016/j.cld.2014.01.007]

43   Polski JM, Brunt EM, Saeed ZA. Chronology of histological changes after band ligation of esophageal varices in humans. *Endoscopy* 2001; **33**: 443-447 [PMID: 11396765 DOI: 10.1055/s-2001-14259]

44   Helmy A, Hayes PC. Review article: current endoscopic therapeutic options in the management of variceal bleeding. *Aliment Pharmacol Ther* 2001; **15**: 575-594 [PMID: 11328251 DOI: 10.1046/j.1365-2036.2001.00950.x]

45   Vanbiervliet G, Giudicelli-Bornard S, Piche T, Berthier F, Gelsi E, Filippi J, Anty R, Arab K, Huet PM, Hebuterne X, Tran A. Predictive factors of bleeding related to post-banding ulcer following endoscopic variceal ligation in cirrhotic patients: a case-control study. *Aliment Pharmacol Ther* 2010; **32**: 225-232 [PMID: 20412065 DOI: 10.1111/j.1365-2036.2010.04331.x]

46   Krag A, Wiest R, Albillos A, Gluud LL. The window hypothesis: haemodynamic and non-haemodynamic effects of β-blockers improve survival of patients with cirrhosis during a window in the disease. *Gut* 2012; **61**: 967-969 [PMID: 22234982 DOI: 10.1136/gutjnl-2011-301348]

47   Pérez-Paramo M, Muñoz J, Albillos A, Freile I, Portero F, Santos M, Ortiz-Berrocal J. Effect of propranolol on the factors promoting bacterial translocation in cirrhotic rats with ascites. *Hepatology* 2000; **31**: 43-48 [PMID: 10613726 DOI: 10.1002/hep.510310109]

48   Pérez-Ayuso RM, Piqué JM, Bosch J, Panés J, González A, Pérez R, Rigau J, Quintero E, Valderrama R, Viver J. Propranolol in prevention of recurrent bleeding from severe portal hypertensive gastropathy in cirrhosis. *Lancet* 1991; **337**: 1431-1434 [PMID: 1675316 DOI: 10.1016/0140-6736(91)93125-S]

49   Senzolo M, Cholongitas E, Burra P, Leandro G, Thalheimer U, Patch D, Burroughs AK. beta-Blockers protect against spontaneous bacterial peritonitis in cirrhotic patients: a meta-analysis. *Liver Int* 2009; **29**: 1189-1193 [PMID: 19508620 DOI: 10.1111/j.1478-3231.2009.02038.x]

50   Cholongitas E, Papatheodoridis GV, Manesis EK, Burroughs AK, Archimandritis AJ. Spontaneous bacterial peritonitis in cirrhotic patients: Is prophylactic propranolol therapy beneficial? *J Gastroenterol Hepatol* 2006; **21**: 581-587 [PMID: 16638103 DOI: 10.1111/j.1440-1746.2005.03982.x]

51   Hoshino S, Shinoura S, Akamine H, Kikuchi K, Keida Y. Effect of propranolol for the prevention of spontaneous bacterial peritonitis. *Am J Gastroenterol* 2000; **95**: 2513 [DOI: 10.1111/j.1572-0241.2000.02710.x]

52   Ge PS, Runyon BA. The changing role of beta-blocker therapy

in patients with cirrhosis. *J Hepatol* 2014; **60**: 643-653 [PMID: 24076364 DOI: 10.1016/j.jhep.2013.09.016]

53   van Buuren HR, Rasch MC, Batenburg PL, Bolwerk CJ, Nicolai JJ, van der Werf SD, Scherpenisse J, Arends LR, van Hattum J, Rauws EA, Schalm SW. Endoscopic sclerotherapy compared with no specific treatment for the primary prevention of bleeding from esophageal varices. A randomized controlled multicentre trial [ISRCTN03215899]. *BMC Gastroenterol* 2003; **3**: 22 [PMID: 12919638 DOI: 10.1186/1471-230X-3-22]

54   Baroncini D, Milandri GL, Borioni D, Piemontese A, Cennamo V, Billi P, Dal Monte PP, D'Imperio N. A prospective randomized trial of sclerotherapy versus ligation in the elective treatment of bleeding esophageal varices. *Endoscopy* 1997; **29**: 235-240 [PMID: 9255524 DOI: 10.1055/s-2007-1004182]

55   Avgerinos A, Armonis A, Stefanidis G, Mathou N, Vlachogiannakos J, Kougioumtzian A, Triantos C, Papaxoinis C, Manolakopoulos S, Panani A, Raptis SA. Sustained rise of portal pressure after sclerotherapy, but not band ligation, in acute variceal bleeding in cirrhosis. *Hepatology* 2004; **39**: 1623-1630 [PMID: 15185303 DOI: 10.1002/hep.20236]

56   European Association for the Study of the Liver. EASL Clinical Practice Guidelines: Vascular diseases of the liver. *J Hepatol* 2016; **64**: 179-202 [PMID: 26516032 DOI: 10.1016/j.jhep.2015.07.040]

57   Qi X, Han G, Fan D. Management of portal vein thrombosis in liver cirrhosis. *Nat Rev Gastroenterol Hepatol* 2014; **11**: 435-446 [PMID: 24686266 DOI: 10.1038/nrgastro.2014.36]

58   Han G, Qi X, Guo W, Niu J, Bai M, Fan D. Transjugular intrahepatic portosystemic shunt for portal vein thrombosis in cirrhosis. *Gut* 2012; **61**: 326-327 [PMID: 21757449 DOI: 10.1136/gutjnl-2011-300577]

59   Han G, Qi X, He C, Yin Z, Wang J, Xia J, Yang Z, Bai M, Meng X, Niu J, Wu K, Fan D. Transjugular intrahepatic portosystemic shunt for portal vein thrombosis with symptomatic portal hypertension in liver cirrhosis. *J Hepatol* 2011; **54**: 78-88 [PMID: 20932597 DOI: 10.1016/j.jhep.2010.06.029]

60   Luca A, Miraglia R, Caruso S, Milazzo M, Sapere C, Maruzzelli L, Vizzini G, Tuzzolino F, Gridelli B, Bosch J. Short- and long-term effects of the transjugular intrahepatic portosystemic shunt on portal vein thrombosis in patients with cirrhosis. *Gut* 2011; **60**: 846-852 [PMID: 21357252 DOI: 10.1136/gut.2010.228023]

61   Qi X, He C, Guo W, Yin Z, Wang J, Wang Z, Niu J, Bai M, Yang Z, Fan D, Han G. Transjugular intrahepatic portosystemic shunt for portal vein thrombosis with variceal bleeding in liver cirrhosis: outcomes and predictors in a prospective cohort study. *Liver Int* 2016; **36**: 667-676 [PMID: 26235541 DOI: 10.1111/liv.12929]

62   Senzolo M, Tibbals J, Cholongitas E, Triantos CK, Burroughs AK, Patch D. Transjugular intrahepatic portosystemic shunt for portal vein thrombosis with and without cavernous transformation. *Aliment Pharmacol Ther* 2006; **23**: 767-775 [PMID: 16556179 DOI: 10.1111/j.1365-2036.2006.02820.x]

63   Zheng M, Chen Y, Bai J, Zeng Q, You J, Jin R, Zhou X, Shen H, Zheng Y, Du Z. Transjugular intrahepatic portosystemic shunt versus endoscopic therapy in the secondary prophylaxis of variceal rebleeding in cirrhotic patients: meta-analysis update. *J Clin Gastroenterol* 2008; **42**: 507-516 [PMID: 18344888 DOI: 10.1097/MCG.0b013e31815576e6]

64   Zhang H, Zhang H, Li H, Zhang H, Zheng D, Sun CM, Wu J. TIPS versus endoscopic therapy for variceal rebleeding in cirrhosis: A meta-analysis update. *J Huazhong Univ Sci Technolog Med Sci* 2017; **37**: 475-485 [PMID: 28786052 DOI: 10.1007/s11596-017-1760-6]

65   Harras F, Sheta el S, Shehata M, El Saadany S, Selim M, Mansour L. Endoscopic band ligation plus argon plasma coagulation versus scleroligation for eradication of esophageal varices. *J Gastroenterol Hepatol* 2010; **25**: 1058-1065 [PMID: 20594219 DOI: 10.1111/j.1440-1746.2010.06265.x]

66   Petersen B, Barkun A, Carpenter S, Chotiprasidhi P, Chuttani R, Silverman W, Hussain N, Liu J, Taitelbaum G, Ginsberg GG; Technology Assessment Committee, American Society for Gastrointestinal Endoscopy. Tissue adhesives and fibrin glues. *Gastrointest Endosc* 2004; **60**: 327-333 [PMID: 15332018 DOI: 10.1016/S0016-5107(04)01564-0]

67   **El Amin H**, Abdel Baky L, Sayed Z, Abdel Mohsen E, Eid K, Fouad Y, El Khayat H. A randomized trial of endoscopic variceal ligation versus cyanoacrylate injection for treatment of bleeding junctional varices. *Trop Gastroenterol* 2010; **31**: 279-284 [PMID: 21568143]

68   **Evrard S**, Dumonceau JM, Delhaye M, Golstein P, Devière J, Le Moine O. Endoscopic histoacryl obliteration vs. propranolol in the prevention of esophagogastric variceal rebleeding: a randomized trial. *Endoscopy* 2003; **35**: 729-735 [PMID: 12929019 DOI: 10.1055/s-2003-41581]

69   **Abraldes JG**, Villanueva C, Aracil C, Turnes J, Hernandez-Guerra M, Genesca J, Rodriguez M, Castellote J, García-Pagán JC, Torres F, Calleja JL, Albillos A, Bosch J; BLEPS Study Group. Addition of Simvastatin to Standard Therapy for the Prevention of Variceal Rebleeding Does Not Reduce Rebleeding but Increases Survival in Patients With Cirrhosis. *Gastroenterology* 2016; **150**: 1160-1170.e3 [PMID: 26774179 DOI: 10.1053/j.gastro.2016.01.004]

70   **Sinagra E**, Perricone G, D'Amico M, Tinè F, D'Amico G. Systematic review with meta-analysis: the haemodynamic effects of carvedilol compared with propranolol for portal hypertension in cirrhosis. *Aliment Pharmacol Ther* 2014; **39**: 557-568 [PMID: 24461301 DOI: 10.1111/apt.12634]

71   **Bhardwaj A**, Kedarisetty CK, Vashishtha C, Bhadoria AS, Jindal A, Kumar G, Choudhary A, Shasthry SM, Maiwall R, Kumar M, Bhatia V, Sarin SK. Carvedilol delays the progression of small oesophageal varices in patients with cirrhosis: a randomised placebo-controlled trial. *Gut* 2017; **66**: 1838-1843 [PMID: 27298379 DOI: 10.1136/gutjnl-2016-311735]

72   **Li T**, Ke W, Sun P, Chen X, Belgaumkar A, Huang Y, Xian W, Li J, Zheng Q. Carvedilol for portal hypertension in cirrhosis: systematic review with meta-analysis. *BMJ Open* 2016; **6**: e010902 [PMID: 27147389 DOI: 10.1136/bmjopen-2015-010902]

73   **Bosch J**. Carvedilol for portal hypertension in patients with cirrhosis. *Hepatology* 2010; **51**: 2214-2218 [PMID: 20513005 DOI: 10.1002/hep.23689]

74   **Villanueva C**, Graupera I, Aracil C, Alvarado E, Miñana J, Puente Á, Hernandez-Gea V, Ardevol A, Pavel O, Colomo A, Concepción M, Poca M, Torras X, Reñe JM, Guarner C. A randomized trial to assess whether portal pressure guided therapy to prevent variceal rebleeding improves survival in cirrhosis. *Hepatology* 2017; **65**: 1693-1707 [PMID: 28100019 DOI: 10.1002/hep.29056]

75   **Qi X**, Méndez-Sánchez N, Mancuso A, Romeiro FG, Guo X. Who should receive endoscopic variceal ligation after recovering from acute variceal bleeding? *Hepatology* 2018; **67**: 2057-2058 [PMID: 29171864 DOI: 10.1002/hep.29684]

CASE REPORT

Gastric adenocarcinoma of fundic gland type with signet-ring cell carcinoma component: A case report and review of the literature

Keita Kai, Masaaki Satake, Osamu Tokunaga

Keita Kai, Department of Pathology, Saga University Hospital, Saga 849-8501, Japan

Masaaki Satake, Department of Gastroenterology, Koga Hospital 21, Kurume 839-0801, Japan

Osamu Tokunaga, Department of Pathology, Shin Koga Hospital, Kurume 830-8577, Japan

ORCID number: Keita Kai (0000-0003-1553-2598); Masaaki Satake (0000-0003-2264-8687); Osamu Tokunaga (0000-0002-4976-1148).

Abstract

A depressed lesion was found at a gastric angle of 76-year-old Japanese woman by esophagogastroduodenoscopy. Four years prior, she was diagnosed with a *Helicobacter pylori* infection but no eradication was performed. The pathological diagnosis of biopsy specimens was signet-ring cell carcinoma. Endoscopic submucosal dissection (ESD) was performed. Histopathological examination of the ESD specimen revealed proliferation of well-differentiated tubular adenocarcinoma mimicking fundic gland cells at the deep layer of the lamina propria mucosae. These tumor cells expressed focally pepsinogen-I, diffusely MUC6, and scattered H$^+$/K$^+$ ATPase according to immunohistochemistry. Therefore, we diagnosed this tumor as gastric adenocarcinoma of fundic gland type (GA-FG). Adjacent to the GA-FG, proliferation of signet-ring cell carcinoma which diffusely expressed MUC 2 and MUC 5AC was observed. Intestinal metaplasia was focally observed in the surrounding mucosa of the signet-ring cell carcinoma. To the best of our knowledge, this is the first case report of GA-FG with a signet-ring cell carcinoma component. The origin of signet-ring cell carcinoma, *i.e.*, whether it accidentally arose from a non-neoplastic mucosa and coexisted with the GA-FG or dedifferentiated from the GA-FG is unclear at present. We expect the accumulation of similar cases and further analysis to clarify this issue.

Key words: Gastric adenocarcinoma of fundic gland type; Endoscopic submucosal dissection; *Helicobacter pylori*; Intestinal metaplasia; Signet-ring cell carcinoma

Core tip: Gastric adenocarcinoma of fundic gland type is a very rare variant of a well-differentiated gastric adenocarcinoma. To the best of our knowledge, this is the first case report of gastric adenocarcinoma of fundic gland type with a signet-ring cell carcinoma component.

INTRODUCTION

Gastric adenocarcinoma showing chief cell differentiation was initially reported by Tsukamoto *et al*[1] in 2007. In 2010, Ueyama *et al*[2] reported 10 cases of gastric adenocarcinoma showing chief cell differentiation which expressed pepsinogen-Ⅰ (a marker for chief cells) and proposed the concept of gastric adenocarcinoma of fundic gland type (GA-FG). Since then, the concept of GA-FG has been widely recognized, and reported cases and studies have been gradually accumulated.

Because GA-FG is thought to originate from the gastric mucosa of the fundic gland region without chronic gastritis or intestinal metaplasia, it has been generally considered that GA-FG develops without *Helicobacter pylori* (*H. pylori*) infection[3]. However, cases of GA-FG with current *H. pylori* infection or post-eradiation therapy were recently reported[4,5]. GA-FG generally presents as a well-differentiated adenocarcinoma with mild nuclear atypia and is generally considered to have a low potential for malignancy, although an extremely rare case of advanced GA-FG showing high-grade malignancy was reported[6]. To the best of our knowledge, no GA-FG case with a poorly differentiated adenocarcinoma or signet-ring cell carcinoma component has been reported.

We recently encountered a case of GA-FG with a signet-ring carcinoma component which developed in a patient with current *H. pylori* infection, and we report the case as follows.

CASE REPORT

A 76-year-old Japanese woman visited a nearby clinic complaining of a dull feeling in the stomach. Esophagogastroduodenoscopy (EGD) revealed a depressed lesion at a gastric angle of the greater curvature side. She was referred to our hospital for further examination. She had been found to have an *H. pylori* infection by a urease test four years ago, but no eradication was performed. The depressed lesion was confirmed by an EGD performed at Koga Hospital 21 (Figure 1A) and narrow band imaging (Figure 1B) showed a relatively demarcated lesion with an irregular microsurface pattern. A biopsy of the depressed lesion was performed. Histologically, the biopsy specimens consisted of several fragments of gastric mucosa with intestinal metaplasia. Among the glands with intestinal metaplasia, a small number of atypical cells showing a signet-ring-cell-like appearance were found (Figure 1C). As these atypical cells were positive for immunohistochemistry of pan-cytokeratin (AE1/AE3), a pathological diagnosis of signet-ring cell carcinoma was made (Figure 1D). Endoscopic submucosal dissection (ESD) was performed.

Pathological findings

The ESD specimen showed a slightly depressed lesion measuring 28 mm × 14 mm. In that lesion, a deeper depressed lesion measuring 12 mm × 3 mm was found. Histologically, a well-differentiated tubular adenocarcinoma mimicking the fundic gland cells, mainly the chief cells, proliferated at the deep layer of the lamina propria mucosae (Figure 2A). The tumor cells had slightly enlarged nuclei and showed mild nuclear atypia. The structure and differentiation toward the surfaces of the fundic gland were significantly disturbed compared to normal fundic glands (Figure 2B). The tumor had invaded into the submucosal layer, and the maximum depth of invasion was 400 μm (Figure 2C). No lymphatic or venous invasion was observed. The mucosal surface was covered with non-neoplastic foveolar epithelium.

Adjacent to the well-differentiated tubular adenocarcinoma mimicking fundic gland cells, proliferation of a signet-ring cell carcinoma producing intra- and extracellular mucin was observed (Figure 2A). Proliferation of the signet-ring cell carcinoma was restricted within the lamina propria mucosae, and no lymphatic or venous invasion was observed. Focally, intestinal metaplasia was observed at the mucosa surrounding the signet-ring cell carcinoma (Figure 2A).

In immunohistochemistry, the tumor cells of well-differentiated tubular adenocarcinoma expressed focally (30%) pepsinogen-Ⅰ (Figure 3A), diffusely MUC6 (Figure 3B) and scattered (5%) H^+/K^+ ATPase (Figure 3C). Therefore, we diagnosed this tumor as GA-FG. The tumor cells of GA-FG were negative for MUC 2 but diffusely positive for MUC 5AC (Figure 3D). Meanwhile, the tumor cells of the signet-ring cell carcinoma were diffusely positive for MUC 2 (Figure 3E) and MUC 5AC (Figure 3F) but negative for pepsinogen-Ⅰ, MUC6, and H^+/K^+ ATPase. The immunohistochemistry results are summarized in Table 1.

Based on these HE and immunohistochemical findings, we made the final diagnosis of GA-FG with a signet-ring cell carcinoma component. The mapping based on histology revealed that GA-FG was distributed at a slightly depressed lesion (28 mm × 14 mm) and the signet-ring cell carcinoma was distributed at a deeper depressed lesion (12 mm × 3 mm) in the slightly depressed lesion
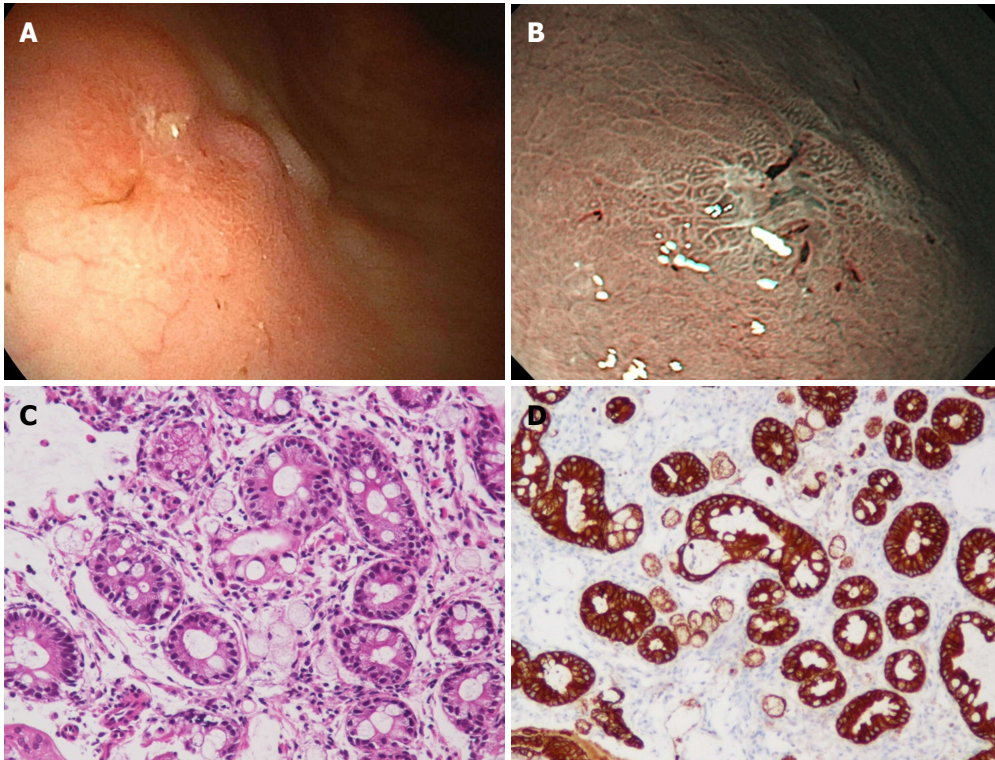
Figure 1 Image from esophagogastroduodenoscopy. A: Depressed lesion was found at gastric angle of the greater curvature side; B: The narrow band imaging of the EGD showed a relatively demarcated lesion with an irregular microsurface pattern; C: The biopsy specimen from the depressed lesion. Among the glands with intestinal metaplasia, a small number of signet-ring cell carcinoma cells were found (HE; × 200). D: Signet-ring cell carcinoma cells were positive for immunohistochemistry of pan-cytokeratin (× 200). EGD: Esophagogastroduodenoscopy.

Figure 2 Pathological findings. A: Representative histological photograph of the specimens of endoscopic submucosal dissection (HE; × 50). Proliferation of gastric adenocarcinoma of the fundic gland type (GA-FG) are observed at the deep layer of the lamina propria mucosae in the left half of the photo (blue dot line: the border of GA-FG). Adjacent to the GA-FG, proliferation of the signet-ring cell carcinoma producing intra- and extracellular mucin is observed in the right half of the photo (yellow dotted line: border of the signet-ring cell carcinoma). Intestinal metaplasia was observed at the mucosa surrounding the signet-ring cell carcinoma (arrows); B: Structure and differentiation toward the surfaces of the fundic gland were significantly disturbed at the GA-FG compared to the normal fundic glands (HE; × 50). The blue line is the border of the GA-FG and the normal fundic glands. The mucosal surface was covered with non-neoplastic foveolar epithelium. Intestinal metaplasia cannot be observed in this photo; C: GA-FG invaded into the submucosal layer (HE; × 100).

Table 1 Results of Immunohistochemistry

	MUC 6	H+/K+ ATPase	Pepsinogen- I	MUC5AC	MUC2
Gastric adenocarcinoma of fundic gland type	+ (Diffuse)	+ (Scattered, 5%)	+ (Focal, 30%)	+ (Diffuse)	-
Signet-ring cell carcinoma	-	-	-	+ (Diffuse)	+ (Diffuse)

Figure 3 Photographs of immunohistochemistry. The magnifications of all photographs are × 200. The tumor cells of GA-FG expressed focally (30%) pepsinogen- I (A), diffusely MUC6 (B), scattered (5%) H+/K+ ATPase (C), and diffusely MUC5AC (D). The tumor cells of the signet-ring cell carcinoma diffusely expressed MUC 2 (E) and MUC 5AC (F). GA-FG: Gastric adenocarcinoma of fundic gland type.

Figure 4 Mapping of the endoscopic submucosal dissection specimen based on histology. GA-FG distributed at a slightly depressed lesion measuring 28 mm × 14 mm (red line) and signet-ring cell carcinoma distributed at a deeper depressed lesion measuring 12 mm × 3 mm in the slightly depressed lesion (yellow line). GA-FG: Gastric adenocarcinoma of fundic gland type.

(Figure 4).

DISCUSSION

GA-FG is a very rare variant of a well-differentiated gastric adenocarcinoma accounting for 1.6% of gastric adenocarcinomas[7]. GA-FGs are characterized by the following: (1) They arise most commonly from the normal gastric mucosa of the fundic gland region without intestinal metaplasia; (2) they are recognized as smooth elevated or depressed lesions; (3) they often invade the submucosal layer, while lymphatic and venous invasion are rare; and (4) the atypia of the tumor cell is usually mild[8]. The Wnt/β-catenin signal signaling pathway and *GNAS* mutations are considered to contribute to the development and progression of GA-FG[7,9,10].

Immunohistochemically, GA-FG variably express the following biomarkers of fundic gland cells: MUC6 for mucous neck cells; H^+/K^+ ATPase for parietal cells; and pepsinogen- I for chief cells. Typical cases diffusely express pepsinogen- I and MUC6 and show scattered positivity for H^+/K^+ ATPase. These cases are referred to as GA-FG of the chief cell predominant type[2]. GA-FGs do not express the intestinal-type mucin of MUC2.

The distinctive feature of present case was the co-existence of the signet-ring cell carcinoma and GA-FG. To the best of our knowledge, no GA-FG case which contains signet-ring cell carcinoma has been reported. The signet-ring cell carcinoma component in our case expressed the

intestinal type of MUC2, and intestinal metaplasia was focally observed in the background mucosa. In addition, the present case had a current *H. pylori* infection. These are unusual findings for GA-FG.

The origin of the signet-ring cell carcinoma is a very interesting subject. We propose two hypotheses regarding this issue. First, these two lesions (GA-FG and the signet-ring cell carcinoma) may have accidentally coexisted. Usually, GA-FGs develop at the fundic gland in a deep layer of the gastric mucosa, and the normal foveolar epithelium remains at the surface. In the present case, intestinal metaplasia due to chronic inflammation caused by the *H. pylori* infection was focally observed at the surface of the mucosa. Therefore, it seems reasonable that the signet-ring cell carcinoma producing intestinal-type mucin developed at the surface of the mucosa from the intestinal metaplasia and that GA-FG simultaneously developed from the fundic gland of the deep layer of the mucosa. However, the probability for this situation to occur is considered extremely low.

The second hypothesis is that a part of the GA-FG dedifferentiated into signet-ring cell carcinoma. Although dedifferentiation or transformation is often observed in various types of malignant tumors, no GA-FG case showing dedifferentiation or transformation has been reported. Usually, GA-FGs do not express MUC5AC, which is a marker of the foveolar epithelium; however, it is known that GA-FGs rarely express MUC5AC[2,8,11]. In the present case, both the GA-FG and signet-ring cell carcinoma expressed MUC5AC. Ueyama *et al*[2] speculated that MUC5AC is only expressed in advanced GA-FG lesions with a large diameter and massive submucosal invasion, suggesting that cell differentiation changes from the fundic gland type to the foveolar type during disease progression. This speculation regarding MUC5AC seems to support the potential for the transformation of GA-FG. However, we believe it is impossible to conclusively determine the origin of the signet-ring cell carcinoma in the present case because of a lack of reliable evidence.

In conclusion, we have reported the first case of GA-FG with a signet-ring cell carcinoma component which expressed an intestinal type of mucin. Our case had a current *H. pylori* infection and showed focal intestinal metaplasia in the background mucosa. The origin of the signet-ring cell carcinoma is unclear at present. We expect the accumulation of the similar cases and further analysis of whether dedifferentiation or transformation can really occur in GA-FG.

ACKNOWLEDGMENTS

ARTICLE HIGHLIGHTS

Case characteristics
A 76-year-old Japanese woman visited a nearby clinic complaining of a dull feeling in the stomach.

Clinical diagnosis
Esophagogastroduodenoscopy (EGD) revealed a depressed lesion at a gastric angle of the greater curvature side.

Differential diagnosis
The clinical diagnosis of early gastric cancer was considered by EGD findings.

Laboratory diagnosis
No specific finding was obtained by laboratory testing.

Imaging diagnosis
The narrow band imaging of EGD showed a relatively demarcated lesion with an irregular microsurface pattern.

Pathological diagnosis
Pathological findings of endoscopic submucosal dissection (ESD) specimens indicated the diagnosis of gastric adenocarcinoma of fundic gland type (GA-FG) with a signet-ring cell carcinoma component.

Treatment
Only ESD was performed for treatment.

Related reports
To the best of our knowledge, no GA-FG case with a poorly differentiated adenocarcinoma or signet-ring cell carcinoma component has been reported.

Term explanation
The term GA-FG describes gastric adenocarcinoma of fundic gland type.

Experiences and lessons
This is the first case report of GA-FG with a signet-ring cell carcinoma component.

REFERENCES

1   **Tsukamoto T**, Yokoi T, Maruta S, Kitamura M, Yamamoto T, Ban H, Tatematsu M. Gastric adenocarcinoma with chief cell differentiation. *Pathol Int* 2007; **57**: 517-522 [PMID: 17610477 DOI: 10.1111/j.1440-1827.2007.02134.x]

2   **Ueyama H**, Yao T, Nakashima Y, Hirakawa K, Oshiro Y, Hirahashi M, Iwashita A, Watanabe S. Gastric adenocarcinoma of fundic gland type (chief cell predominant type): proposal for a new entity of gastric adenocarcinoma. *Am J Surg Pathol* 2010; **34**: 609-619 [PMID: 20410811 DOI: 10.1097/PAS.0b013e3181d94d53]

3   **Miyazawa M**, Matsuda M, Yano M, Hara Y, Arihara F, Horita Y, Matsuda K, Sakai A, Noda Y. Gastric adenocarcinoma of the fundic gland (chief cell-predominant type): A review of endoscopic and clinicopathological features. *World J Gastroenterol* 2016; **22**: 10523-10531 [PMID: 28082804 DOI: 10.3748/wjg.v22.i48.10523]

4   **Chiba T**, Kato K, Masuda T, Ohara S, Iwama N, Shimada T, Shibuya D. Clinicopathological features of gastric adenocarcinoma of the fundic gland (chief cell predominant type) by retrospective and prospective analyses of endoscopic findings. *Dig Endosc* 2016; **28**: 722-730 [PMID: 27129734 DOI: 10.1111/den.12676]

5   **Manabe S**, Mukaisho KI, Yasuoka T, Usui F, Matsuyama T, Hirata I,

Boku Y, Takahashi S. Gastric adenocarcinoma of fundic gland type spreading to heterotopic gastric glands. *World J Gastroenterol* 2017; **23**: 7047-7053 [PMID: 29097877 DOI: 10.3748/wjg.v23.i38.7047]

6   **Ueo T**, Yonemasu H, Ishida T. Gastric adenocarcinoma of fundic gland type with unusual behavior. *Dig Endosc* 2014; **26**: 293-294 [PMID: 24321002 DOI: 10.1111/den.12212]

7   **Hidaka Y**, Mitomi H, Saito T, Takahashi M, Lee SY, Matsumoto K, Yao T, Watanabe S. Alteration in the Wnt/β-catenin signaling pathway in gastric neoplasias of fundic gland (chief cell predominant) type. *Hum Pathol* 2013; **44**: 2438-2448 [PMID: 24011952 DOI: 10.1016/j.humpath.2013.06.002]

8   **Miyazawa M**, Matsuda M, Yano M, Hara Y, Arihara F, Horita Y, Matsuda K, Sakai A, Noda Y. Gastric adenocarcinoma of fundic gland type: Five cases treated with endoscopic resection. *World J Gastroenterol* 2015; **21**: 8208-8214 [PMID: 26185396 DOI: 10.3748/wjg.v21.i26.8208]

9   **Murakami T**, Mitomi H, Yao T, Saito T, Shibuya T, Watanabe S. Epigenetic regulation of Wnt/β-catenin signal-associated genes in gastric neoplasia of the fundic gland (chief cell-predominant) type. *Pathol Int* 2017; **67**: 147-155 [PMID: 28105693 DOI: 10.1111/pin.12509]

10   **Kushima R**, Sekine S, Matsubara A, Taniguchi H, Ikegami M, Tsuda H. Gastric adenocarcinoma of the fundic gland type shares common genetic and phenotypic features with pyloric gland adenoma. *Pathol Int* 2013; **63**: 318-325 [PMID: 23782334 DOI: 10.1111/pin.12070]

11   **Ueyama H**, Matsumoto K, Nagahara A, Hayashi T, Yao T, Watanabe S. Gastric adenocarcinoma of the fundic gland type (chief cell predominant type). *Endoscopy* 2014; **46**: 153-157 [PMID: 24338239 DOI: 10.1055/s-0033-1359042]

Baishideng®

26>

9 771007 932045

World Journal of

Gastroenterology

Contents

Contents

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, C. Mel Wilcox, MD, Doctor, Professor, Division of Gastroenterology and Hepatology, University of Alabama at Birmingham, Birmingham, AL 35294, United States

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 642 experts in gastroenterology and hepatology from 59 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

EDITORS FOR THIS ISSUE

EDITORIAL

Indocyanine green-based fluorescence imaging in visceral and hepatobiliary and pancreatic surgery: State of the art and future directions

Gian Luca Baiocchi, Michele Diana, Luigi Boni

Gian Luca Baiocchi, Department of Clinical and Experimental Sciences, University of Brescia, Brescia 25125, Italy

Michele Diana, IHU-Strasbourg, Institute of Image-Guided Surgery, Strasbourg 67000, France

Michele Diana, IRCAD, Research Institute against Cancer of the Digestive System, Strasbourg 67000, France

Luigi Boni, General and Emergency Surgery, IRCCS - Ca' Granda - Policlinico Hospital, Milan 20122, Italy

ORCID number: Gian Luca Baiocchi (0000-0003-2402-2178); Michele Diana (0000-0002-1390-8486); Luigi Boni (0000-0002-5906-3810).

Author contributions: Baiocchi GL and Boni L conceived the study; Baiocchi GL, Diana M, and Boni L critically reviewed the literature and supplied the material to be included in the manuscript's Figures; Baiocchi GL drafted the manuscript; all authors approved the final version of the article.

Conflict-of-interest statement: Baiocchi GL, Diana M, and Boni L have received no funding and declare no conflicts of interest in relation to this specific work. Baiocchi GL received a travel grant from Stryker and from Karl Storz and has been the scientific organizer of the international workshop "Intraoperative ICG Fluorescence Imaging in Hepatobiliary and Visceral Surgery: State of the Art and New Frontiers," (Brescia, Italy, October 21, 2017) partly funded (travel expenses) by Karl Storz and Stryker companies. Diana M is the recipient of a grant from the ARC Foundation (Project ELIOS) to develop fluorescence image-guided surgery. Boni L is a consultant for Storz and Olympus.

Abstract

In recent years, the use of fluorescence-guided surgery (FGS) to treat benign and malignant visceral, hepatobiliary and pancreatic neoplasms has significantly increased. FGS relies on the fluorescence signal emitted by injected substances (fluorophores) after being illuminated by ad hoc laser sources to help guide the surgical procedure and provide the surgeon with real-time visualization of the fluorescent structures of interest that would be otherwise invisible. This review surveys and discusses the most common and emerging clinical applications of indocyanine green (ICG)-based fluorescence in visceral, hepatobiliary and pancreatic surgery. The analysis, findings, and discussion presented here rely on the authors' significant experience with this technique in their medical institutions, an up-to-date review of the most relevant articles published on this topic between 2014 and 2018, and lengthy discussions with key opinion leaders in the field during recent conferences and congresses. For each application, the benefits and limitations of this technique, as well as applicable future directions, are described. The imaging of fluorescence emitted by ICG is a simple, fast,

relatively inexpensive, and harmless tool with numerous different applications in surgery for both neoplasms and benign pathologies of the visceral and hepatobiliary systems. The ever-increasing availability of visual systems that can utilize this tool will transform some of these applications into the standard of care in the near future. Further studies are needed to evaluate the strengths and weaknesses of each application of ICG-based fluorescence imaging in abdominal surgery.

Key words: Indocyanine green; Fluorescence imaging; Gastrointestinal surgery; Liver surgery; Biliary surgery; Pancreatic surgery; Visceral perfusion; Biliary anatomy; Peritoneal carcinomatosis

Core tip: In recent years, the use of fluorescence-guided surgery to treat benign and malignant visceral, hepatobiliary and pancreatic neoplasms has significantly increased. It helps guide the surgical procedure and provides the surgeon with real-time visualization of the fluorescent structures of interest that would be otherwise invisible. This review surveys and discusses the most common and emerging clinical applications of indocyanine green-based fluorescence in visceral, hepatobiliary and pancreatic surgery. The ever-increasing availability of visual systems that can utilize this tool will transform some of these applications into the standard of care in the near future.

INTRODUCTION

Fluorescence-guided surgery (FGS) is a modality of intraoperative navigation that provides the surgeon with enhanced visualization of anatomical structures and/or an improved understanding of an organ's current perfusion in real time[1,2]. To obtain a fluorescence signal, the operative field is exposed to near-infrared light sources, while the target is injected with a fluorescent dye, which can emit a fluorescence signal after being excited by ad hoc laser sources. The fluorescence signal can be visualized either directly on the operative field during open surgical procedures or captured by specific cameras and displayed on a screen in a minimally invasive setting. FGS is currently more adapted to the needs of surgical navigation because it does not require bulky equipment in the operating room and the enhanced information provided to the surgeon

is displayed in real time, without interfering with the surgical workflow[3].

In the last few years, fluorescence imaging using the fluorescent properties of indocyanine green (ICG) has become widespread in several medical and surgical specialties[4]. Due to its relatively low cost and high availability, ICG is the most employed fluorophore in the clinical setting of general surgery.

The main goal of this overview is to survey and discuss the most recent and ongoing clinical applications of ICG-based fluorescence in visceral, hepatobiliary and pancreatic (HBP) surgery. Up to date, vascular assessment of anastomotic stump in visceral surgery and extrahepatic biliary anatomy identification during difficult cholecystectomies are the most well established applications of ICG-based fluorescence surgery.

The in-depth analysis presented here relies on three main sources of information. First, the analysis draws on the authors' significant experience with this technology in their medical institutions. Second, an up-to-date review of the most relevant articles published on this topic between 2014 and 2018 presents the latest developments in this rapidly developing field. Lastly, lengthy discussions of the authors with key opinion leaders in the field during recent conferences and congresses add novel insights on the ongoing applications that will appear in the literature in the near future. In particular, the rationale of applying this technique to visceral and HBP surgery, the relevant physiopathological insights, the available data, and the ongoing clinical trials are thoroughly surveyed and discussed. For each application, the benefits and limitations of this technique are identified, and applicable future directions are described.

REAL-TIME VISCERAL PERFUSION

The estimation of real-time visceral perfusion is a promising intraoperative application of ICG-based fluorescence surgery. ICG-based fluorescence angiography might guide the identification of the optimal resection site and help estimate the blood supply of visceral anastomosis in both upper GI and colorectal surgery. The rationale behind fluorescence angiography is that the fluorescent dye, upon systemic injection, should reach and highlight only vascularized areas.

Focusing on colorectal surgery, it is well known that the rate of anastomotic leaks ranges between 5% and 7%, even in high-volume centers[5]. The introduction into daily clinical practice of a tool capable of delivering optimal stump vascularization could reduce this rate. In turn, this may eliminate dehiscence due to inadequate vascularization despite a macroscopically satisfactory appearance[6-8]. Some phase II trials have confirmed the feasibility, low cost, and high success rate of this procedure[9], reporting a leakage rate (< 3%) lower than the one expected on the basis of historical series in rectal anastomoses. The rate of changes in the

transection line varies from 5% to 40%[10]. However, a few reports did not confirm these data; for instance, in a series of 346 colorectal interventions, Kin and colleagues reported a leakage rate of 7.5% and 6.4% with and without ICG intraoperative assessment, respectively[11]. Some randomized prospective controlled trials are currently ongoing[12].

Similar use of ICG for intraoperative assessments has been investigated for esophagectomy and gastrectomy. When making the gastric tube during esophagectomy, the most cranial portion represents a critical point because the gastroepiploic vascularization reaches a macroscopically unclear cut-off at that level[13]. A few retrospective, case-control studies have confirmed this hypothesis, showing a number of changes in the site of anastomosis as high as 25% and a reduction in the leakage rate from 18% to 3% and from 15% to 6% in the series reported by Karampinis and Kitagawa, respectively[14,15]. In a consecutive series of 40 patients undergoing the Ivor-Lewis procedure, Koyanagi *et al*[16] compared the blood flow using ICG in the gastric conduit and in the greater curve; by employing ROC analysis, they identified a speed of 1.76 cm/s as the cut-off value predicting leakage. However, even in esophageal surgery, the lack of prospective randomized trials hampers the possibility of reaching a definitive conclusion supporting evaluation of the perfusion of the gastric conduit using ICG-based fluorescence.

The same principles for checking the perfusion of the upper extremity of the stomach transection line apply to sleeve gastrectomy in bariatric surgery. Identifying the less vascularized proximal area would allow a targeted resection and reduce the leakage rate along the suture line[17].

In many other clinical settings, the estimation of visceral perfusion may turn out to be a useful and relatively inexpensive intraoperative tool, for example, to assess the small bowel trophism during urgent interventions for intestinal ischemia, after mistaken mesocolon interruption, and/or small bowel mesenteric division[18].

Identification of the major vascular structures in the early phase of dissection from the surrounding fat might be an additional and potentially very useful development from a theoretical point of view. Small intravenous boluses of ICG can facilitate navigation of the vascular structure. However, the penetration depth of the fluorescence is limited to 5-6 mm; furthermore, after the injection, the tissues surrounding both the large arteries and veins become fluorescent, causing a background fluorescence field that reduces visualization. Finally, from then on, the bowel exhibits background staining, which could reduce the clarity of stump perfusion as evaluated at the time of anastomosis. For these reasons, repeated intraoperative angiographies for visualizing the main vascular structure are not possible with ICG.

BILIARY ANATOMY

ICG-based fluorescence imaging to visualize and study biliary anatomy represents one of the most established applications of this technique in abdominal surgery. The reason is simple: the exclusively hepatic metabolism of ICG, which results in biliary excretion starting approximately 30 min after i.v. injection, enables clear visualization of the biliary anatomy, which is very useful during difficult cholecystectomies[19] and from a theoretical point of view during the resection of centrally located liver tumors and hilar (Klatskin's) tumors[20].

There is more than one comparative study that, although always retrospective, enables us to conclude that ICG-based fluorescence imaging is an extremely useful tool when applied to visualize and study the biliary anatomy[21]. In a systematic review published in 2017, 19 studies were analyzed. There was moderate-quality evidence that ICG-assisted visualization of the extrahepatic bile duct is superior to intraoperative cholangiogram (RR = 1.16; 95%CI: 1.00-1.35)[22]. While the difference was not significant in that study, it was significant in some other single-center series[23]. Considering the relative ease and speed of the procedure, in the future, ICG-based visualization of the biliary anatomy might replace intraoperative cholangiography, which requires a preliminary dissection of the confluence between the cystic and bile ducts and cannulation of the structures.

Recently, in an experimental study with a limited number of cases (7 pigs), ICG injection directly into the gallbladder enabled superior biliary anatomy visualization compared to i.v. injection[24]. The same study group translated their analysis to patients in a subsequent series of 46 cases[25].

With the aim of overcoming the limits of retrospective data, a prospective randomized controlled trial (FALCON) was set up in 2016 to compare near-infrared fluorescence cholangiography-assisted laparoscopic cholecystectomy versus conventional laparoscopic cholecystectomy. The results are expected in 2 years[26].

The main point of debate remains the ideal injection time. Although fluorescence of the bile duct can be detected shortly after i.v. injection, the high fluorescence of the liver parenchyma makes it less evident in contrast. In our view, the ideal timing for this injection is 3 to 5 h before surgery. However, a recent series demonstrated that an injection 24 h before surgery could still be possible[27].

LIVER SURGERY

In liver surgery, the state of the art still considers ICG mainly as a simple reagent for the preoperative evaluation of hepatic function. The comparatively limited use of ICG-based fluorescence imaging in liver surgery with respect to other surgical specialties is slightly paradoxical, considering the pathophysiological

assumption that ICG exhibits only hepatic metabolism and only bile excretion. In addition, many centers devoted to liver surgery use the ICG clearance rate as a reliable liver function indicator[28,29].

During the development of fluorescence cholangiography, it was discovered that ICG accumulates in the cancerous tissues of hepatocellular carcinoma (HCC) and in the noncancerous hepatic parenchyma around adenocarcinoma foci. The first application of this imaging technique was reported in 2009 by Ishizawa *et al*[30] and Gotoh *et al*[31].

From a theoretical point of view, three remarks should be taken into consideration. First, in HCC (which obviously contains hepatocytes), ICG can be captured and retained by malignant cells; moreover, the secretion of ICG may be altered due to architectural disorders reducing the possibility of excretion[32]. Second, if the tumor is formed by non-hepatocellular cells, as in the case of metastases, ICG could be retained by a group of hepatocytes surrounding the nodule and compressed by the nodule itself[33]. Third, if the nodule contains predominantly epithelial cells that are normally part of the biliary ducts (cholangiocarcinoma), they should not absorb the dye and should behave in a manner similar to metastases but with greater alterations in biliary delivery processes[34].

In clinical practice, however, these theoretical insights may not be taken into consideration because there is currently no clearly defined timing for i.v. injection. Indeed, especially at an early stage, increased vascularization of the neoplasm could result in greater exposure to the dye, regardless of the type of cells contained therein. The nodule behavior in the portal and tardive phases (with more or less washout) is also relevant in determining the amount of retained ICG. These factors translate into the absence of widely accepted guidelines for the intraoperative use of ICG-based fluorescence to differentiate benign from malignant nodules and among the different types of malignant tumors.

The key objective of ongoing studies (some of which are being performed in our medical institutions) is to enable differential diagnosis. ICG-based fluorescence may then be extremely helpful, as follows: (1) In the detection of small superficial malignant nodules not detected in the preoperative study (especially for managing metastases)[35]; and (2) in excluding the malignant nature of small superficial nodules of uncertain contrast medium behavior (especially useful for HCC in cirrhotic liver tissue)[36].

To date, however, some consolidated uses in liver surgery exist. First, it is common practice that metastases in healthy livers are well visualized by the presence of an ICG capture ring due to the compression of the surrounding cells. This can be an advantage during laparoscopic surgery, where tactile sensation is not available as a guide for resection, and continuous monitoring with intraoperative ultrasound is both difficult

and time consuming[37]. The presence of the nontumor tissue ring can also be a useful indicator of the extent of resection required to obtain an adequate free margin[38].

Second, there is an increasing need to treat patients with liver metastases after they have undergone multiple chemotherapy cycles; in these cases, nontumor parenchyma is hardened and metastases are largely necrotic, and, hence, softer. For this reason, the normal, easy tactile perception of metastasis is more difficult, and finding a fluorescent area can be of great help.

The third application pertains to some primary liver neoplasms without a capsule, for which it is difficult to assess the optimum extent of the resection. In these cases, a good rule should be to check the absence of complete residual fluorescence at the end of the surgical transection.

Fourth, there are neoplasms with peripheral biliary infiltration and peribiliary spreading, which are not clearly recognizable by preoperative study, except for data indicating a dilatation of the upstream biliary structures[39]. In these cases, ICG retention affects the whole area that drains the Glisson segment and not just the nodule, suggesting the need for a wider resection.

Moreover, some authors have assessed the use of ICG for detecting biliary leaks in the hepatic transection surface after liver resection[40]. Finally, experimental studies involving intraportal and intrahepatic artery ICG injection (by celiac trunk catheterization in experimental models and by femoral puncture in hybrid operating rooms) have been published with the aim of achieving precise, real-time definitions of liver segmentation[41-43].

The lesion depth represents the major limitation in applying ICG-based fluorescence imaging to liver surgery. Further technological improvements are needed to deepen the fluorescence penetration more than the current 5-6 millimeters[44,45]. In addition, few data exist regarding extrahepatic HCC metastases, but this may represent a promising clinical application for FGS[46].

ONCOLOGICAL SURGERY OF THE UPPER AND LOWER GI TRACTS

The simplest use of ICG to surgically treat cancers affecting the digestive tract consists of endoscopically marking tumors to see the tumor site, which is necessary for small and nonserous infiltrating cancers. In this context, ICG is similar to other dyes used to date (China, indigo carmine)[47,48].

However, the most intriguing and potentially powerful application of FGS is currently one involving nodal navigation in esophageal, gastric, and colorectal cancers[49]. Injection of the dye into peritumoral tissues, ideally performed endoscopically in the submucosa, should allow for the intraoperative evaluation of lymphatic diffusion in real time. Multicentric prospective series are already available on the use of ICG to identify the sentinel lymph node in gastric cancer.
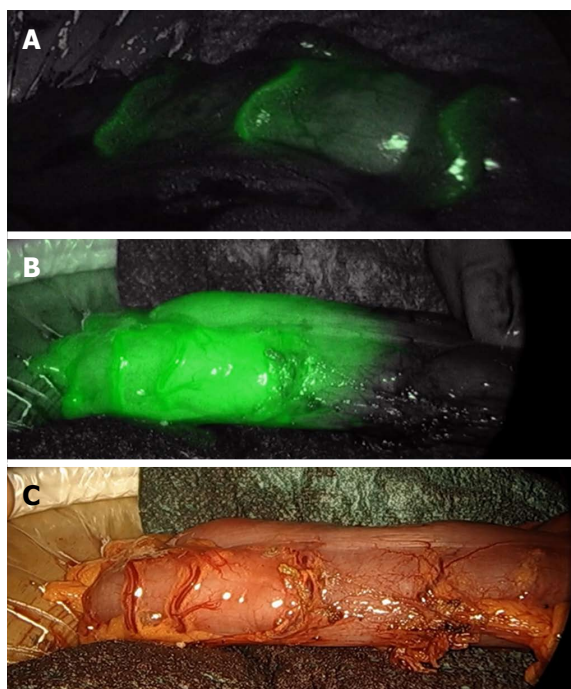
Figure 1 Colon perfusion before anastomosis during left colectomy. A few seconds after the i.v. injection of 0.3 mg/kg indocyanine green, bowel arteries clearly appear (A); thereafter, the bowel perfusion cut-off area becomes evident (B and C).

These studies demonstrate that ICG is superior to both radioactive tracers and dyestuffs used to date[50,51]. In a recent multicentric Japanese series of 44 EGCs, the identification of sentinel node basins and the accuracy of metastatic node identification were both 100%[52].

The same strategy has been used for assessing colorectal tumors, investigating both the sentinel node basin and the enlarged mesocolic excision area draining the tumor site[53,54]. This latter principle is indeed the opposite of the sentinel node strategy: the goal would be to remove all lymph nodes that have received ICG as possible metastatic sites originating from the specific tumor site. The method is primarily useful for anatomical studies. Indeed, the ideal injection timing for the visualization of lymph nodes is not yet clear. Likely, after many hours, the dye may pass local lymph node drainage stations and move up to the central lymphatic pathways. The ideal study should therefore include (1) a preoperative tumor injection at different times before surgery up to an intraoperative injection; (2) the subsequent removal of individual lymph nodes; (3) a fluorescence evaluation for each node; and (4) a comparison of the final histological data.

In clinical practice, the problems are mainly related to the feasibility of the injection. For cancer of the esophagus, stomach, and rectum, the injection can be endoscopic and intraoperative (despite causing an increase in time and costs), while in the proximal colon, it is easier to proceed with a serous injection during the initial stages of the intervention. Clinical experience shows that the injection should be limited to a few cc,

as staining rapidly spreads in lymphatic and adipose tissues. If, during the injection, a few drops of dye come out of the serosa on the peritoneum, they stain the surrounding structures and the surgical instruments and cannot be washed away.

Despite the above limitations, the possible applications of this method for navigating lymph nodes appear to be of great interest and have very important clinical consequences. It is clearly emerging that the ideal lymphadenectomy needs to be individualized based on preoperative staging. However, in many cases, imaging is not able to discern with high probability, for example, between T1 and T2. On the one hand, limiting lymphadenectomy to stations of real potential interest could allow considerable time savings and reduce most of the major postoperative complications, often related to vascular lesions secondary to an excessive nodal dissection. On the other hand, for example, in rectal surgery, extending lymphadenectomy to stations, such as obturatory and inguinal nodes, that are not usually removed could reduce the rate of lymph node recurrence[55].

The same applies to periaortic stations in cardia and proximal stomach cancer, to the right colic artery basin in cecum and ascending colon cancer, and to flexural cancers. A small series of 10 hepatic and 10 splenic flexure colon cancers reported a 25% rate of changes to the mesocolon resection line due to ICG lymphatic flow visualization[56]. A subsequent series of 31 patients with splenic flexure cancer from the same study group confirmed that all metastatic nodes were included in the area determined by ICG lymphatic flow visualization and that interesting insights into the lymphatic spread of those tumors can be derived[57].

In the near future, it is important to search for a factor predicting the positivity of perigastric and extraperigastric nodes based on their ICG load. To date, a relationship between ICG quantities in dissected nodes and the likelihood of the nodes bearing metastases has not been demonstrated. Studies focusing on the sentinel node strategy have shown that the radioisotope count was a significant predictive factor for metastasis[58]. In contrast, some very limited series recently described patients with unexpected nodal metastases from colorectal cancer discovered by fluorescence visualization after i.v. ICG injection while searching for peritoneal carcinomatosis[59]; this report suggests that a particular trophism of intravenously injected ICG for cancer cells may be discovered.

Few studies have taken into account the same type of lymph node navigation in pancreatic cancers, which have extremely rich and complex lymph node spread patterns. A recent series of 10 patients undergoing laparoscopic pancreaticoduodenectomy for periampullary cancers with i.v. ICG injection during retroportal fat tissue dissection revealed a possible advantage in obtaining an R0 margin at this level[60]. However, at this stage, it is not possible to hypothesize and assess the clinical usefulness
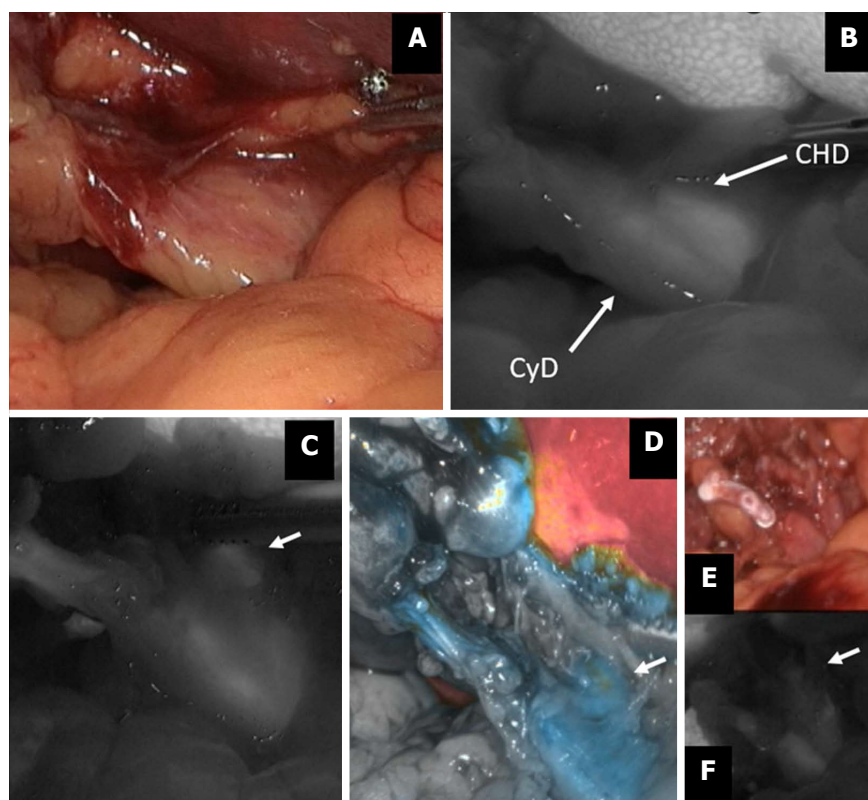
Figure 2 Indocyanine green-enhanced biliary anatomy. During a difficult cholecystectomy for acute cholecystitis (A), the confluence between the cystic duct (CyD) and the common hepatic duct (CHD) is shown by fluorescence imaging (B); common hepatic duct (arrow) is further visualized before (C and D) and after (E and F) cystic duct division. ICG: Indocyanine green.

of this strategy in pancreatic surgery[61]. Interestingly, a small series of pancreatic neuroendocrine tumors (NETs) has demonstrated a clear affinity of ICG for NET cells, which enables the removal of some residual tissue at the end of the first resection in laparoscopic distal pancreatectomy[62].

PERITONEAL CARCINOMATOSIS

This is an area where few studies have been conducted[63]. Peritoneal carcinomatosis remains a mode of metastatic diffusion that is largely devoid of real therapeutic strategies. Thus, finding it at an early stage while it is too small to be visible to the human eye, could represent a major clinical breakthrough. Some current studies are trying to link ICG to epithelial cell markers that should not be present on the peritoneum, which contains only mesothelial cells. No clinical outcomes of these studies are yet available, but some experimental settings have been developed in animal models. For instance, Cheng and colleagues have shown that a surgical navigation system combining optical molecular targets with an ICG-linked molecular probe is able to identify a peritoneal carcinomatosis site as small as 1.8 mm[64]. On the other hand, reports of some very limited series have shown that after i.v. injection, ICG is retained by cancerous cells in the peritoneum[65,66]. There is no clear explanation for these clinical observations, which, if confirmed in

larger studies, may have very high clinical value as a comprehensive modality for staging visceral cancers.

CONCLUSION AND PERSPECTIVE

The key take-home messages from this overview are as follows. (1) The most well-established use of fluorescence imaging is for checking anastomotic stump perfusion in visceral surgery (Figure 1); Some prospective uncontrolled series and retrospective controlled studies, mainly dealing with colorectal and esophageal surgery, have confirmed the usefulness of this technique; (2) The second field of application for which there is a unanimous consensus in the literature pertains to visualization of the biliary anatomy during cholecystectomy (Figure 2). A comparison with intraoperative cholangiography demonstrated the superiority of fluorescence in terms of simplicity, timing, and efficacy; (3) In liver surgery, multiple possible uses of fluorescence have been described (tumor visualization, especially in laparoscopic surgery, vascular segmentation, biliary leak identification) (Figure 3). However, no comparative studies are available; (4) With regard to lymph node navigation in gastrointestinal tumors, most studies in the literature have focused on the sentinel node technique, whereas the use of fluorescence for complete lymphadenectomy has been described only by a few very experienced centers (Figure 4); and (5) Finally, there are some interesting

Figure 3 Indocyanine green in liver surgery. Primary liver tumors show intense and complete staining because their hepatocytes take up ICG but do not secrete it (A and B); liver metastases show a ring appearance because their cells do not take up ICG but hepatocytes surrounding the nodule are compressed (C and D). ICG: Indocyanine green.

Figure 4 Indocyanine green fluorescence imaging in extended right hemicolectomy. The figure displays the right branches of middle colic vessel division during extended right hemicolectomy for transverse colon cancer. ICG injected in the tumor site spreads in nodes at the very proximal root of the artery. ICG fluorescence imaging allows a radical lymphadenectomy, including very small nodes (A and B). Only when all the stained nodes are removed may the nodal dissection be considered radical (C). ICG: Indocyanine green.

preliminary findings regarding the use of fluorescence for the early detection of peritoneal carcinomatosis.

An important issue pertains to the complexity and costs of this technology. The major positive feature is that the required training is minimal and the learning curve is extremely rapid, because the visualization is activated by either clicking a button on the camera or hitting a pedal. When considering the costs of ICG-based fluorescence imaging, the relevant distinction is between the fixed costs of the equipment versus the variable costs of the injections. The highest costs lie in obtaining a NIR fluorescence camera system with dedicated optical devices, cameras, and light cables. Depending on the system chosen (goggles, handheld, or stand alone), the cost of the system varies from several thousands to hundreds of thousands of USD (and similarly in other currencies). Nowadays, many major companies sell these tools; therefore, at the fast pace at which cost-saving technological change and updating are occurring, it is very likely that in the next 5 to 10 years most operating theaters will be equipped with the technology required to display the ICG-generated fluorescence. Once the NIR fluorescence camera system is available in a hospital, the additional cost of each surgical procedure utilizing ICG-based fluorescence imaging is relatively limited: as a matter of example, in Italy, Japan, and the United States, a 25 mg vial of ICG costs 80 euros, 588 yen, and 65 USD, respectively[41].

The imaging of fluorescence emitted by ICG is a simple, fast, and relatively inexpensive tool without side effects that has numerous different applications in surgery not only for treating cancers affecting the visceral and hepatobiliary systems but also for visualizing the biliary tree during difficult cholecystectomies. The fast pace at which technological development is occurring in this area and the ever-increasing availability of more powerful visual systems that can utilize this tool will transform some of these applications into the standard of care in the near future.

ACKNOWLEDGMENTS

REFERENCES

1   **Diana M**. Enabling precision digestive surgery with fluorescence imaging. *Transl Gastroenterol Hepatol* 2017; **2**: 97 [PMID: 29264435 DOI: 10.21037/tgh.2017.11.06]

2   **Gioux S**, Choi HS, Frangioni JV. Image-guided surgery using invisible near-infrared light: fundamentals of clinical translation. *Mol Imaging* 2010; **9**: 237-255 [PMID: 20868625]

3   **Nguyen QT**, Tsien RY. Fluorescence-guided surgery with live molecular navigation--a new cutting edge. *Nat Rev Cancer* 2013; **13**: 653-662 [PMID: 23924645 DOI: 10.1038/nrc3566]

4   **Reinhart MB**, Huntington CR, Blair LJ, Heniford BT, Augenstein VA. indocyanine green: historical context, current applications, and future considerations. *Surg Innov* 2016; **23**: 166-175 [PMID: 26359355 DOI: 10.1177/1553350615604053]

5   **Starker PM**, Chinn B. Using outcomes data to justify instituting new technology: a single institution's experience. *Surg Endosc* 2018; **32**: 1586-1592 [PMID: 29273871 DOI: 10.1007/s00464-017-6001-3]

6   **Gossedge G**, Vallance A, Jayne D. Diverse applications for near infra-red intraoperative imaging. *Colorectal Dis* 2015; **17** Suppl 3: 7-11 [PMID: 26394736 DOI: 10.1111/codi.13023]

7   **Boni L**, Fingerhut A, Marzorati A, Rausei S, Dionigi G, Cassinotti E. Indocyanine green fluorescence angiography during laparoscopic low anterior resection: results of a case-matched study. *Surg Endosc* 2017; **31**: 1836-1840 [PMID: 27553790 DOI: 10.1007/s00464-016-5181-6]

8   **Mangano A**, Gheza F, Chen LL, Minerva EM, Giulianotti PC. Indocyanine green (Icg)-enhanced fluorescence for intraoperative assessment of bowel microperfusion during laparoscopic and robotic colorectal surgery: The quest for evidence-based results. *Surg Technol Int* 2018; **32**: 101-104 [PMID: 29611153]

9   **Ris F**, Liot E, Buchs NC, Kraus R, Ismael G, Belfontali V, Douissard J, Cunningham C, Lindsey I, Guy R, Jones O, George B, Morel P, Mortensen NJ, Hompes R, Cahill RA; near-infrared anastomotic perfusion assessment network VOIR. Multicentre phase II trial of near-infrared imaging in elective colorectal surgery. *Br J Surg* 2018; Epub ahead of print [PMID: 29663330 DOI: 10.1002/bjs.10844]

10 **Hellan M**, Spinoglio G, Pigazzi A, Lagares-Garcia JA. The influence of fluorescence imaging on the location of bowel transection during robotic left-sided colorectal surgery. *Surg Endosc* 2014; **28**: 1695-1702 [PMID: 24385249 DOI: 10.1007/s00464-013-3377-6]

11 **Kin C**, Vo H, Welton L, Welton M. Equivocal effect of intraoperative fluorescence angiography on colorectal anastomotic leaks. *Dis Colon Rectum* 2015; **58**: 582-587 [PMID: 25944430 DOI: 10.1097/DCR.0000000000000320]

12 **Sujatha-Bhaskar S**, Jafari MD, Stamos MJ. The role of fluorescent angiography in anastomotic leaks. *Surg Technol Int* 2017; **30**: 83-88 [PMID: 28277591]

13 **Kumagai Y**, Ishiguro T, Haga N, Kuwabara K, Kawano T, Ishida H. Hemodynamics of the reconstructed gastric tube during esophagectomy: assessment of outcomes with indocyanine green fluorescence. *World J Surg* 2014; **38**: 138-143 [PMID: 24196170 DOI: 10.1007/s00268-013-2237-9]

14 **Karampinis I**, Ronellenfitsch U, Mertens C, Gerken A, Hetjens S, Post S, Kienle P, Nowak K. Indocyanine green tissue angiography affects anastomotic leakage after esophagectomy. A retrospective, case-control study. *Int J Surg* 2017; **48**: 210-214 [PMID: 29146267 DOI: 10.1016/j.ijsu.2017.11.00]

15 **Kitagawa H**, Namikawa T, Iwabu J, Fujisawa K, Uemura S, Tsuda S, Hanazaki K. Assessment of the blood supply using the indocyanine green fluorescence method and postoperative endoscopic evaluation of anastomosis of the gastric tube during esophagectomy. *Surg Endosc* 2018; **32**: 1749-1754 [PMID: 28916846 DOI: 10.1007/s00464-017-5857-6]

16 **Koyanagi K**, Ozawa S, Oguma J, Kazuno A, Yamazaki Y, Ninomiya Y, Ochiai H, Tachimori Y. Blood flow speed of the gastric conduit assessed by indocyanine green fluorescence: New predictive evaluation of anastomotic leakage after esophagectomy. *Medicine* (Baltimore) 2016; **95**: e4386 [PMID: 27472732 DOI: 10.1097/MD.0000000000004386]

17 **Frattini F**, Lavazza M, Mangano A, Amico F, Rausei S, Rovera F, Boni L, Dionigi G. Indocyanine green-enhanced fluorescence in laparoscopic sleeve gastrectomy. *Obes Surg* 2015; **25**: 949-950 [PMID: 25736231 DOI: 10.1007/s11695-015-1640-8]

18 **Irie T**, Matsutani T, Hagiwara N, Nomura T, Fujita I, Kanazawa

Y, Kakinuma D, Uchida E. Successful treatment of non-occlusive mesenteric ischemia with indocyanine green fluorescence and open-abdomen management. *Clin J Gastroenterol* 2017; **10**: 514-518 [PMID: 28956274 DOI: 10.1007/s12328-017-0779-3]

19   **Ishizawa T**, Bandai Y, Ijichi M, Kaneko J, Hasegawa K, Kokudo N. Fluorescent cholangiography illuminating the biliary tree during laparoscopic cholecystectomy. *Br J Surg* 2010; **97**: 1369-1377 [PMID: 20623766 DOI: 10.1002/bjs.7125]

20   **Ashitate Y**, Stockdale A, Choi HS, Laurence RG, Frangioni JV. Real-time simultaneous near-infrared fluorescence imaging of bile duct and arterial anatomy. *J Surg Res* 2012; **176**: 7-13 [PMID: 21816414 DOI: 10.1016/j.jss.2011.06.027]

21   **Pesce A**, Piccolo G, La Greca G, Puleo S. Utility of fluorescent cholangiography during laparoscopic cholecystectomy: A systematic review. *World J Gastroenterol* 2015; **21**: 7877-7883 [PMID: 26167088 DOI: 10.3748/wjg.v21.i25.7877]

22   **Vlek SL**, van Dam DA, Rubinstein SM, de Lange-de Klerk ESM, Schoonmade LJ, Tuynman JB, Meijerink WJHJ, Ankersmit M. Biliary tract visualization using near-infrared imaging with indocyanine green during laparoscopic cholecystectomy: results of a systematic review. *Surg Endosc* 2017; **31**: 2731-2742 [PMID: 27844236 DOI: 10.1007/s00464-016-5318-7]

23   **Osayi SN**, Wendling MR, Drosdeck JM, Chaudhry UI, Perry KA, Noria SF, Mikami DJ, Needleman BJ, Muscarella P 2nd, Abdel-Rasoul M, Renton DB, Melvin WS, Hazey JW, Narula VK. Near-infrared fluorescent cholangiography facilitates identification of biliary anatomy during laparoscopic cholecystectomy. *Surg Endosc* 2015; **29**: 368-375 [PMID: 24986018 DOI: 10.1007/s00464-014-3677-5]

24   **Liu YY**, Kong SH, Diana M, Lègner A, Wu CC, Kameyama N, Dallemagne B, Marescaux J. Near-infrared cholecysto-cholangiography with indocyanine green may secure cholecystectomy in difficult clinical situations: proof of the concept in a porcine model. *Surg Endosc* 2016; **30**: 4115-4123 [PMID: 26511116 DOI: 10.1007/s00464-015-4608-9]

25   **Liu YY**, Liao CH, Diana M, Wang SY, Kong SH, Yeh CN, Dallemagne B, Marescaux J, Yeh TS. Near-infrared cholecystocholangiography with direct intragallbladder indocyanine green injection: preliminary clinical results. *Surg Endosc* 2018; **32**: 1506-1514 [PMID: 28916859 DOI: 10.1007/s00464-017-5838-9]

26   **van den Bos J**, Schols RM, Luyer MD, van Dam RM, Vahrmeijer AL, Meijerink WJ, Gobardhan PD, van Dam GM, Bouvy ND, Stassen LP. Near-infrared fluorescence cholangiography assisted laparoscopic cholecystectomy versus conventional laparoscopic cholecystectomy (FALCON trial): study protocol for a multicentre randomised controlled trial. *BMJ Open* 2016; **6**: e011668 [PMID: 27566635 DOI: 10.1136/bmjopen-2016-011668]

27   **Verbeek FP**, Schaafsma BE, Tummers QR, van der Vorst JR, van der Made WJ, Baeten CI, Bonsing BA, Frangioni JV, van de Velde CJ, Vahrmeijer AL, Swijnenburg RJ. Optimization of near-infrared fluorescence cholangiography for open and laparoscopic surgery. *Surg Endosc* 2014; **28**: 1076-1082 [PMID: 24232054 DOI: 10.1007/s00464-013-3305-9]

28   **Hiwatashi K**, Ueno S, Sakoda M, Iino S, Minami K, Mori S, Kita Y, Baba K, Kurahara H, Mataki Y, Maemura K, Shinchi H, Natsugoe S. The evaluation of liver function and surgical influence by ICGR15 after chemotherapy for colorectal liver metastases. *J Cancer* 2016; **7**: 595-599 [PMID: 27053958 DOI: 10.7150/jca.13759]

29   **Levesque E**, Martin E, Dudau D, Lim C, Dhonneur G, Azoulay D. Current use and perspective of indocyanine green clearance in liver diseases. *Anaesth Crit Care Pain Med* 2016; **35**: 49-57 [PMID: 26477363 DOI: 10.1016/j.accpm.2015.06.006]

30   **Ishizawa T**, Fukushima N, Shibahara J, Masuda K, Tamura S, Aoki T, Hasegawa K, Beck Y, Fukayama M, Kokudo N. Real-time identification of liver cancers by using indocyanine green fluorescent imaging. *Cancer* 2009; **115**: 2491-2504 [PMID: 19326450 DOI: 10.1002/cncr.24291]

31   **Gotoh K**, Yamada T, Ishikawa O, Takahashi H, Eguchi H, Yano M, Ohigashi H, Tomita Y, Miyamoto Y, Imaoka S. A novel image-guided surgery of hepatocellular carcinoma by indocyanine green fluorescence imaging navigation. *J Surg Oncol* 2009; **100**: 75-79 [PMID: 19301311 DOI: 10.1002/jso.21272]

32   **Kaibori M**, Matsui K, Ishizaki M, Iida H, Sakaguchi T, Tsuda T, Okumura T, Inoue K, Shimada S, Ohtsubo S, Kusano M, Ikehara Y, Ozeki E, Kitawaki T, Kon M. Evaluation of fluorescence imaging with indocyanine green in hepatocellular carcinoma. *Cancer Imaging* 2016; **16**: 6 [PMID: 27052371 DOI: 10.1186/s40644-016-0064-6]

33   **Shimada S**, Ohtsubo S, Ogasawara K, Kusano M. Macro- and microscopic findings of ICG fluorescence in liver tumors. *World J Surg Oncol* 2015; **13**: 198 [PMID: 26055754 DOI: 10.1186/s12957-015-0615-5]

34   **Lim C**, Vibert E, Azoulay D, Salloum C, Ishizawa T, Yoshioka R, Mise Y, Sakamoto Y, Aoki T, Sugawara Y, Hasegawa K, Kokudo N. Indocyanine green fluorescence imaging in the surgical management of liver cancers: current facts and future implications. *J Visc Surg* 2014; **151**: 117-124 [PMID: 24461273 DOI: 10.1016/j.jviscsurg.2013.11.003]

35   **Peloso A**, Franchi E, Canepa MC, Barbieri L, Briani L, Ferrario J, Bianco C, Quaretti P, Brugnatelli S, Dionigi P, Maestri M. Combined use of intraoperative ultrasound and indocyanine green fluorescence imaging to detect liver metastases from colorectal cancer. *HPB* (Oxford) 2013; **15**: 928-934 [PMID: 23458105 DOI: 10.1111/hpb.12057]

36   **Abo T**, Nanashima A, Tobinaga S, Hidaka S, Taura N, Takagi K, Arai J, Miyaaki H, Shibata H, Nagayasu T. Usefulness of intraoperative diagnosis of hepatic tumors located at the liver surface and hepatic segmental visualization using indocyanine green-photodynamic eye imaging. *Eur J Surg Oncol* 2015; **41**: 257-264 [PMID: 25447030 DOI: 10.1016/j.ejso.2014.09.008]

37   **Terasawa M**, Ishizawa T, Mise Y, Inoue Y, Ito H, Takahashi Y, Saiura A. Applications of fusion-fluorescence imaging using indocyanine green in laparoscopic hepatectomy. *Surg Endosc* 2017; **31**: 5111-5118 [PMID: 28455774 DOI: 10.1007/s00464-017-5576-z]

38   **Zhang YM**, Shi R, Hou JC, Liu ZR, Cui ZL, Li Y, Wu D, Shi Y, Shen ZY. Liver tumor boundaries identified intraoperatively using real-time indocyanine green fluorescence imaging. *J Cancer Res Clin Oncol* 2017; **143**: 51-58 [PMID: 27629877 DOI: 10.1007/s00432-016-2267-4]

39   **Harada N**, Ishizawa T, Muraoka A, Ijichi M, Kusaka K, Shibasaki M, Yamamoto K, Hasegawa K, Bandai Y, Kokudo N. Fluorescence navigation hepatectomy by visualization of localized cholestasis from bile duct tumor infiltration. *J Am Coll Surg* 2010; **210**: e2-e6 [PMID: 20510795 DOI: 10.1016/j.jamcollsurg.2010.02.052]

40   **Kaibori M**, Ishizaki M, Matsui K, Kwon AH. Intraoperative indocyanine green fluorescent imaging for prevention of bile leakage after hepatic resection. *Surgery* 2011; **150**: 91-98 [PMID: 21514613 DOI: 10.1016/j.surg.2011.02.011]

41   **Kobayashi Y**, Kawaguchi Y, Kobayashi K, Mori K, Arita J, Sakamoto Y, Hasegawa K, Kokudo N. Portal vein territory identification using indocyanine green fluorescence imaging: Technical details and short-term outcomes. *J Surg Oncol* 2017; **116**: 921-931 [PMID: 28695566 DOI: 10.1002/jso.24752]

42   **Diana M**, Liu YY, Pop R, Kong SH, Legnèr A, Beaujeux R, Pessaux P, Soler L, Mutter D, Dallemagne B, Marescaux J. Superselective intra-arterial hepatic injection of indocyanine green (ICG) for fluorescence image-guided segmental positive staining: experimental proof of the concept. *Surg Endosc* 2017; **31**: 1451-1460 [PMID: 27495341 DOI: 10.1007/s00464-016-5136-y]

43   **Ueno M**, Hayami S, Sonomura T, Tanaka R, Kawai M, Hirono S, Okada KI, Yamaue H. Indocyanine green fluorescence imaging techniques and interventional radiology during laparoscopic anatomical liver resection (with video). *Surg Endosc* 2018; **32**: 1051-1055 [PMID: 29273869 DOI: 10.1007/s00464-017-5997-8]

44   **Benedicenti S**, Molfino S, Alfano MS, Molteni B, Porsio P, Portolani N, Baiocchi GL. Indocyanine green fluorescence-guided liver resection of metastasis from squamous cell carcinoma invading the biliary tree. *Case Reports Gastrointest Med* 2018; In press

45   **Majlesara A**, Golriz M, Hafezi M, Saffari A, Stenau E, Maier-Hein

L, Müller-Stich BP, Mehrabi A. Indocyanine green fluorescence imaging in hepatobiliary surgery. *Photodiagnosis Photodyn Ther* 2017; **17**: 208-215 [PMID: 28017834 DOI: 10.1016/j.pdpdt.2016.12.005]

46   **Satou S**, Ishizawa T, Masuda K, Kaneko J, Aoki T, Sakamoto Y, Hasegawa K, Sugawara Y, Kokudo N. Indocyanine green fluorescent imaging for detecting extrahepatic metastasis of hepatocellular carcinoma. *J Gastroenterol* 2013; **48**: 1136-1143 [PMID: 23179608 DOI: 10.1007/s00535-012-0709-6]

47   **Watanabe M**, Murakami M, Ozawa Y, Yoshizawa S, Matsui N, Aoki T. Intraoperative identification of colonic tumor sites using a near-infrared fluorescence endoscopic imaging system and indocyanine green. *Dig Surg* 2017; **34**: 495-501 [PMID: 28219066 DOI: 10.1159/000458450]

48   **Nagata J**, Fukunaga Y, Akiyoshi T, Konishi T, Fujimoto Y, Nagayama S, Yamamoto N, Ueno M. Colonic marking with near-infrared, light-emitting, diode-activated indocyanine green for laparoscopic colorectal surgery. *Dis Colon Rectum* 2016; **59**: e14-e18 [PMID: 26734978 DOI: 10.1097/DCR.0000000000000542]

49   **Kusano M**, Tajima Y, Yamazaki K, Kato M, Watanabe M, Miwa M. Sentinel node mapping guided by indocyanine green fluorescence imaging: a new method for sentinel node navigation surgery in gastrointestinal cancer. *Dig Surg* 2008; **25**: 103-108 [PMID: 18379188 DOI: 10.1159/000121905]

50   **Kinami S**, Oonishi T, Fujita J, Tomita Y, Funaki H, Fujita H, Nakano Y, Ueda N, Kosaka T. Optimal settings and accuracy of indocyanine green fluorescence imaging for sentinel node biopsy in early gastric cancer. *Oncol Lett* 2016; **11**: 4055-4062 [PMID: 27313740 DOI: 10.3892/ol.2016.4492]

51   **Tummers QR**, Boogerd LS, de Steur WO, Verbeek FP, Boonstra MC, Handgraaf HJ, Frangioni JV, van de Velde CJ, Hartgrink HH, Vahrmeijer AL. Near-infrared fluorescence sentinel lymph node detection in gastric cancer: A pilot study. *World J Gastroenterol* 2016; **22**: 3644-3651 [PMID: 27053856 DOI: 10.3748/wjg.v22.i13.3644]

52   **Takahashi N**, Nimura H, Fujita T, Mitsumori N, Shiraishi N, Kitano S, Satodate H, Yanaga K. Laparoscopic sentinel node navigation surgery for early gastric cancer: a prospective multicenter trial. *Langenbecks Arch Surg* 2017; **402**: 27-32 [PMID: 27999935 DOI: 10.1007/s00423-016-1540-y]

53   **Cahill RA**, Anderson M, Wang LM, Lindsey I, Cunningham C, Mortensen NJ. Near-infrared (NIR) laparoscopy for intraoperative lymphatic road-mapping and sentinel node identification during definitive surgical resection of early-stage colorectal neoplasia. *Surg Endosc* 2012; **26**: 197-204 [PMID: 21853392 DOI: 10.1007/s00464-011-1854-3]

54   **Sartori CA**, Dal Pozzo A, Franzato B, Balduino M, Sartori A, Baiocchi GL. Laparoscopic total mesorectal excision for rectal cancer: experience of a single center with a series of 174 patients. *Surg Endosc* 2011; **25**: 508-514 [PMID: 20607560 DOI: 10.1007/s00464-010-1202-z]

55   **Nishigori N**, Koyama F, Nakagawa T, Nakamura S, Ueda T, Inoue T, Kawasaki K, Obara S, Nakamoto T, Fujii H, Nakajima Y. Visualization of lymph/blood flow in laparoscopic colorectal cancer surgery by ICG fluorescence imaging (Lap-IGFI). *Ann Surg Oncol* 2016; **23** Suppl 2: S266-S274 [PMID: 25801355 DOI: 10.1245/s10434-015-4509-0]

56   **Watanabe J**, Ota M, Suwa Y, Ishibe A, Masui H, Nagahori K. Real-time indocyanine green fluorescence imaging-guided complete mesocolic excision in laparoscopic flexural colon cancer surgery. *Dis Colon Rectum* 2016; **59**: 701-705 [PMID: 27270525 DOI: 10.1097/DCR.0000000000000608]

57   **Watanabe J**, Ota M, Suwa Y, Ishibe A, Masui H, Nagahori K. Evaluation of lymph flow patterns in splenic flexural colon cancers using laparoscopic real-time indocyanine green fluorescence imaging. *Int J Colorectal Dis* 2017; **32**: 201-207 [PMID: 27695977 DOI: 10.1007/s00384-016-2669-4]

58   **Kamiya S**, Takeuchi H, Nakahara T, Niihara M, Nakamura R, Takahashi T, Wada N, Kawakubo H, Saikawa Y, Omori T, Murakami K, Kitagawa Y. Auxiliary diagnosis of lymph node metastasis in early gastric cancer using quantitative evaluation of sentinel node radioactivity. *Gastric Cancer* 2016; **19**: 1080-1087 [PMID: 26621524 DOI: 10.1007/s10120-015-0572-2]

59   **Liberale G**, Bourgeois P, Larsimont D, Moreau M, Donckier V, Ishizawa T. Indocyanine green fluorescence-guided surgery after IV injection in metastatic colorectal cancer: A systematic review. *Eur J Surg Oncol* 2017; **43**: 1656-1667 [PMID: 28579357 DOI: 10.1016/j.ejso.2017.04.015]

60   **Rho SY**, Kim JS, Chong JU, Hwang HK, Yoon DS, Lee WJ, Kang CM. Indocyanine green perfusion imaging-guided laparoscopic pancreaticoduodenectomy: Potential application in retroperitoneal margin dissection. *J Gastrointest Surg* 2018; Epub ahead of print [PMID: 29633118 DOI: 10.1007/s11605-018-3760-7]

61   **Hirono S**, Tani M, Kawai M, Okada K, Miyazawa M, Shimizu A, Uchiyama K, Yamaue H. Identification of the lymphatic drainage pathways from the pancreatic head guided by indocyanine green fluorescence imaging during pancreaticoduodenectomy. *Dig Surg* 2012; **29**: 132-139 [PMID: 22538463 DOI: 10.1159/000337306]

62   **Paiella S**, De Pastena M, Landoni L, Esposito A, Casetti L, Miotto M, Ramera M, Salvia R, Secchettin E, Bonamini D, Manzini G, D'Onofrio M, Marchegiani G, Bassi C. Is there a role for near-infrared technology in laparoscopic resection of pancreatic neuroendocrine tumors? Results of the COLPAN "colour-and-resect the pancreas" study. *Surg Endosc* 2017; **31**: 4478-4484 [PMID: 28374260 DOI: 10.1007/s00464-017-5501-5]

63   **Filippello A**, Porcheron J, Klein JP, Cottier M, Barabino G. Affinity of indocyanine green in the detection of colorectal peritoneal carcinomatosis. *Surg Innov* 2017; **24**: 103-108 [PMID: 27909239 DOI: 10.1177/1553350616681897]

64   **Lieto E**, Auricchio A, Cardella F, Mabilia A, Basile N, Castellano P, Orditura M, Galizia G. Fluorescence-guided surgery in the combined treatment of peritoneal carcinomatosis from colorectal cancer: Preliminary Results and Considerations. *World J Surg* 2018; **42**: 1154-1160 [PMID: 28929277 DOI: 10.1007/s00268-017-4237-7]

65   **Cheng H**, Chi C, Shang W, Rengaowa S, Cui J, Ye J, Jiang S, Mao Y, Zeng C, Huo H, Chen L, Tian J. Precise integrin-targeting near-infrared imaging-guided surgical method increases surgical qualification of peritoneal carcinomatosis from gastric cancer in mice. *Oncotarget* 2017; **8**: 6258-6272 [PMID: 28009982 DOI: 10.18632/oncotarget.14058]

66   **Liberale G**, Vankerckhove S, Caldon MG, Ahmed B, Moreau M, Nakadi IE, Larsimont D, Donckier V, Bourgeois P; Group R&D for the Clinical Application of Fluorescence Imaging of the Jules Bordet's Institute. Fluorescence imaging after indocyanine green injection for detection of peritoneal metastases in patients undergoing cytoreductive surgery for peritoneal carcinomatosis from colorectal cancer: a pilot study. *Ann Surg* 2016; **264**: 1110-1115 [PMID: 27828822 DOI: 10.1097/SLA.0000000000001618]

Perioperative thromboprophylaxis in liver transplant patients

Lesley De Pietri, Roberto Montalti, Daniele Nicolini, Roberto Ivan Troisi, Federico Moccheggiani, Marco Vivarelli

Lesley De Pietri, Division of Anaesthesiology and Intensive Care Unit, Department of General Surgery, AUSL Reggio Emilia-IRCCS, Reggio Emilia 42123, Italy

Roberto Montalti, Daniele Nicolini, Federico Moccheggiani, Marco Vivarelli, Hepatobiliary and Abdominal Transplantation Surgery, Department of Experimental and Clinical Medicine, Polytechnic University of Marche, Ancona 60126, Italy

Roberto Ivan Troisi, Department of General, Hepatobiliary and Liver Transplantation Surgery, Ghent University Hospital Medical School, Ghent 185 3K3 9000, Belgium

Roberto Ivan Troisi, Department of Clinical Medicine, Federico II University Naples, Naples 80138, Italy

ORCID number: Lesley De Pietri (0000-0003-4925-9606); Roberto Montalti (0000-0002-3915-3851); Daniele Nicolini (0000-0002-5477-3346); Roberto Ivan Troisi (0000-0001-6280-810X); Federico Moccheggiani (0000-0002-8833-5360); Marco Vivarelli (0000-0003-0500-9461).

Author contributions: De Pietri L designed the literature review strategy, critically planned the article structure and wrote the paper; Montalti R, Nicolini D, Moccheggiani F, Troisi RI and Vivarelli M critically reviewed the article and contributed to the literature review.

Correspondence to: Lesley De Pietri, MD, Staff Physician, Division of Anaesthesiology and Intensive Care Unit, Department of General Surgery, AUSL Reggio Emilia-IRCCS, Viale Risorgimento 80, Reggio Emilia 42123, Italy. lesley.depietri@yahoo.it
Telephone: +39-522-296111

Abstract

Improvements in surgical and anesthetic procedures have increased patient survival after liver transplantation (LT). However, the perioperative period of LT can still be affected by several complications. Among these, thromboembolic complications (intracardiac thrombosis, pulmonary embolism, hepatic artery and portal vein thrombosis) are relatively common causes of increased morbidity and mortality. The benefit of thromboprophylaxis in general surgical patients has already been established, but it is not the standard of care in LT recipients. LT is associated with a high bleeding risk, as it is performed in a setting of already unstable hemostasis. For this reason, the role of routine perioperative prophylactic anticoagulation is usually restricted. However, recent data have shown that the bleeding tendency of cirrhotic patients is not an expression of an acquired bleeding disorder but rather of coexisting factors (portal hypertension, hypervolemia and infections). Furthermore, in cirrhotic patients, the new paradigm of "rebalanced hemostasis" can easily tip towards hypercoagulability because of the recently described enhanced thrombin generation, procoagulant changes in fibrin structure and platelet hyperreactivity. This new coagulation balance, along with improvements in surgical techniques and critical support, has led to a

dramatic reduction in transfusion requirements, and the intraoperative thromboembolic-favoring factors (venous stasis, vessels clamping, surgical injury) have increased the awareness of thrombotic complications and led clinicians to reconsider the limited use of anticoagulants or antiplatelets in the postoperative period of LT.

Key words: Anticoagulation; Liver transplantation; Antiplatelets; Thrombosis; Coagulation; Heparin; Thromboelastography; Thromboprophylaxis; Hepatic artery thrombosis; Portal vein thrombosis

Core tip: The improvements in surgical and anesthetic techniques during liver transplantation (LT) have led to such a reduction in transfusion requirements that bleeding risk is no longer the major concern. The increased knowledge of coagulation balance and the reported incidence of thrombotic complications (hepatic artery and portal vein thrombosis, intracardiac thrombosis, pulmonary embolism) in the LT setting have brought attention to perioperative thromboprophylaxis in an attempt to decrease the morbidity and mortality associated with these complications. The major concern of thromboprophylaxis is the risk of bleeding complications in a setting of already unstable hemostasis. Hence, monitoring its administration and the careful selection of the patients to be treated are of great importance.

INTRODUCTION

Cirrhosis has been traditionally conceived as a hypocoagulable condition, and bleeding is a feared complication of any invasive procedure. In patients with end-stage liver disease, significant coagulopathy (as defined by routine coagulation tests) has historically led clinicians to consider cirrhosis to be the prototype of acquired bleeding disorders, thus supporting the common practice of empirically transfusing patients with blood products or prohemostatic agents to correct standard laboratory test values to reduce the risk of bleeding[1,2]. In this review, we underline the concept of rebalanced hemostasis typical of cirrhotic patients (both in patients with cirrhosis and in patients with acute liver failure), as obtained by the parallel declines in pro- and antihemostatic drivers, resulting in a net hemostatic balance[3]. We also discuss the paradoxical

pro-thrombotic tendency of cirrhotic patients. The new hemostatic balance, in fact, is thought to be much less stable compared with that of healthy patients and can easily tip it towards bleeding or thrombosis.

Prophylactic anticoagulation represents a routine practice to prevent thromboembolic complications [deep vein thrombosis (DVT) and pulmonary embolism (PE)] that can be potentially life-threatening in the postoperative course. Although the utility of thromboprophylaxis after general surgery has been established beyond doubt, in liver transplantation (LT), this opinion is not uniformly shared and does not represent a routine practice. LT is considered an operation with major bleeding risks. Transplanted livers may have delayed primary function, and coagulation does not improve immediately after transplantation, making hemorrhagic complications and transfusions not uncommon[4,5]. In contrast, although intraoperative thrombotic phenomena are uncommon, they are associated with an elevated mortality (ranging from 45% to 68% for PE and 50% for early hepatic artery thrombosis (HAT) and from 32% to 60% for portal vein thrombosis (PVT))[6-9].

Here, we reviewed the literature assessing thrombotic risks in cirrhotic patients and in the post-transplant period and evaluated whether empirical pharmacological thromboprophylaxis strategies and the use of global hemostasis assays may play a beneficial role in reducing this procedure-related thrombotic complications. We do not cover mechanical prophylaxis (intermittent pneumatic compression) because it must be considered and adopted in all abdominal surgery patients who are at a moderate or high risk for venous thromboembolism (VTE), who are, in turn, at a high risk for major bleeding complications[10].

NEW CONCEPT OF REBALANCED HEMOSTASIS AND THE UTILITY OF STANDARD LABORATORY TESTS

A marked reduction of both procoagulant factors (factors II, V, VII, IX, X, XI, XII), anticoagulant factors (anti-thrombin III, protein C, and protein S), an increase in von Willebrand factor (vWF) and a reduced level of ADAMTS13, a vWF-cleaving protease, are the specific features of cirrhosis and bring the patient to a new hemostatic balance[11]. vWF performs its hemostatic functions by binding to factor VIII and to constituents of connective tissue and by promoting platelet adhesion to endothelial surfaces and platelet aggregation under high shear stress[11].

Thrombocytopenia, as a consequence of hypersplenism in patients with portal hypertension, abnormal thrombopoietin metabolism, increased platelet destruction mediated by antiplatelet antibodies, and bone marrow suppression caused by alcohol, antiviral and immunosuppressive therapies, is another clinical feature of chronic liver disease[12]. Unless the platelet

Table 1 Balance of antihemostatic and prohemostatic drivers in the cirrhotic patient

	Anti-hemostatic drivers	Pro-hemostatic drivers
Primary hemostasis	Abnormal platelet function	Elevated vWF
	Thrombocytopenia	Reduced ADAMTS 13
	Decreased production of thrombopoietin	Platelet hyperreactivity
Coagulation	Reduced synthesis of factors II, V, VII, IX, X, and XI	Elevated factor VIII
	Vitamin K deficiency	Reduced anticoagulant protein C, protein S, antithrombin III
	Hypo-dysfibrinogenemia	Procoagulant changes in fibrin structure
Fibrinolysis	Low α2-antiplasmin,	Low plasminogen
	factor XIII, and TAFI	High PAI
	Elevated tPA	

vWF: Von Willebrand factor; TAFI: Thrombin-activatable fibrinolysis inhibitor; t-PA: Tissue plasminogen activator; PAI: Plasminogen activator inhibitor; ADAMTS13: A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13.

count is severely low ($< 50 \times 10^9$/L), thrombocytopenia does not represent an increased bleeding risk. Such a low platelet count is usually sufficient to guarantee a normal thrombin generation, and the low number of platelets is compensated by a higher level of vWF, which is responsible for greater platelet adhesion[13,14]. Hyperfibrinolysis is another described feature of end-stage liver disease, but its role in the coagulopathy of cirrhosis is still debated[15].

Elevated tissue plasminogen activator and a deficiency of thrombin-activatable fibrinolysis inhibitor have been associated with laboratory changes typical of hyperfibrinolysis and an increased risk of bleeding[16]. However, cirrhosis has also been associated with reduced fibrinolysis, as shown by the decreased plasminogen and increased plasminogen activator inhibitor. The contrasting results explain the ongoing debate regarding the absence or presence of a hyperfibrinolytic state in patients with liver disease, even if the balance of fibrinolysis is probably restored by the parallel changes in profibrinolytic and antifibrinolytic drivers[17]. The decreased synthesis of both procoagulants and anticoagulants typical of cirrhosis usually restores a normal hemostatic balance (Table 1), which is so fragile that it can easily become unbalanced in a hemorrhagic or prothrombotic sense, depending on the presence of renal failure, infection, or upper gastrointestinal bleeding. The complexity of hemostatic balance in end-stage liver disease comes from the fact that a patient with cirrhosis and sepsis can be equally at risk for thrombosis, as a result of inflammation[18], and for bleeding, as a result of the release of anticoagulant endogenous heparinoids[19]. The new equilibrium described in stable patients with cirrhosis makes bleeding episodes related more to portal hypertension and hypervolemia than to defective hemostasis.

Despite this "balanced" hemostatic condition, cirrhosis results in the prolongation of standard coagulation tests, which usually do not analyze the complex interplay between pro- and anticoagulants and thus do not provide an accurate evaluation of the alteration in the *in vivo* hemostatic balance[11,20]. Prothrombin time

(PT), activated partial thromboplastin time (aPTT) and International Normalized Ratio (INR) provide only a measure of procoagulant factors and are insensitive to the plasma levels of anticoagulant factors, so they are unreliable to depict the hemostatic status of patients with end-stage liver disease. The lack of reliability of standard plasma coagulation tests, as underlined in the Baveno VI guidelines[21], comes from the fact they are performed without thrombomodulin addition, which is the main protein C activator, and they only detect the first 5% of whole thrombin formation. The normal to increased thrombin generation shown by thrombomodulin-modified thrombin generation tests has further confirmed the unreliability of standard coagulation tests. Therefore, PT, aPTT and INR are untrustworthy in predicting bleeding risk or thrombotic risk and guiding perioperative hemostatic therapy[22]. Platelet number also poorly represents hemostatic capacity in cirrhotic patients, and thrombocytopenia is usually balanced by a marked increase in the plasma level of von Willebrand factor[13]. The defects in antifibrinolytic proteins balanced by decreased plasminogen[23], the decreased fibrinogen partially balanced by prothrombotic changes in the structure of the fibrin clot[24], and the increased factor VIII and reduced antithrombin and protein C constitute the basis for the recently recognized normo- or hypercoagulable state of cirrhosis, with a consequent risk of thromboembolic events.

THROMBOTIC COMPLICATIONS IN MEDICAL CIRRHOTIC PATIENTS AND POTENTIAL ROLE FOR ANTICOAGULATION

Traditionally, the endogenous coagulopathy and thrombocytopenia that characterize cirrhosis made bleeding complications the major concern while managing these patients. The increased knowledge of their coagulation balance and the reported incidence of thrombotic complications in end-stage liver disease patients have recently made the thrombotic risk more feared than

bleeding complications. In 2006, Northup et al[25] found that approximately 0.5% of all admissions involving cirrhosis patients resulted in a new thromboembolic event. Thus, despite the endogenous coagulopathy of cirrhosis, some patients experience venous thromboembolism, such as DVT or PE. Subsequent studies noted that liver cirrhosis per se represents a risk factor for VTE and that all the conditions that can favor any thrombotic complication are frequent in patients with end-stage liver disease. Platelet hyperreactivity, normal or enhanced thrombin generation, as shown by the thrombin generation test, endothelial dysfunction, hyperdynamic circulation, which can bring to circulation stasis, and the reduced mobility of the fragile cirrhotic patient are recognized prothrombotic conditions. Other authors have shown that this thrombophilia seems to worsen as the liver disease progresses, exposing the cirrhotic patients with more severe disease to a greater risk for VTE[26]. Hypoalbuminemia, as an expression of the severity of liver disease and the degree of portal hypertension, has been associated with an increased rate of VTE[27].

Recently, Ambrosino et al[28] conducted a systematic review and meta-analysis to evaluate the risk of VTE associated with cirrhosis, which was 3.7%. They confirmed a significantly increased VTE risk in 695012 cirrhotic patients compared with 1494660 non-cirrhotic controls (OR: 1.703; 95%CI: 1.333, 2.175; $P < 0.0001$). In particular, patients with cirrhosis experienced an increased prevalence of DVT compared to non-cirrhotic subjects. In contrast, more heterogeneity among studies has been found for PE risk, even if it has been shown to be higher in the cirrhotic population.

PVT, which is rare in the general population but relatively frequent in patients with cirrhosis, is another typical feature of chronic liver disease. Its prevalence increases with the severity of liver disease, ranging from 0.6% to 26%[29]. While patients with compensated cirrhosis are rarely affected, PVT is frequently detected in advanced stages, increasing to 25% in LT candidates and to 35% in cirrhotic patients with hepatocellular carcinoma (HCC)[30]. The main risk factors for PVT are the same as those described for thromboembolic disease in general: hypercoagulability, endothelial lesions, reduced portal blood flow and, when HCC is present, the neoplastic invasion of the portal vein. Because of the high prevalence of this thrombotic complication and the possible repercussions on LT[31], the available data suggest that prophylactic PVT treatment may be indicated in cirrhotic patients awaiting LT or after hepatic resection, even if this medical practice has not yet been included in international guidelines due to a lack of randomized controlled trials[32,33]. According to the Baveno VI consensus, anticoagulation should be considered in potential LT candidates with thrombosis of the main portal trunk or progressive PVT, with the goal of facilitating LT and reducing post-transplant morbidity and mortality[21].

THROMBOEMBOLIC EVENTS IN SURGICAL LIVER TRANSPLANT PATIENTS

Traditionally, perioperative bleeding complications, attributed to the endogenous coagulopathy and thrombocytopenia, represented the major concern during LT. In recent years, thanks to improvements in surgical techniques and anesthetic care, hemorrhagic complications have become less frequent, making transplants without intraoperative transfusion more common. The awareness of the new hemostatic balance that characterizes cirrhotic patients, together with the reduced bleeding complications during surgery, has redirected the attention to perioperative thrombotic complications, making surgeons and anesthetists more aware of the possibility of systemic venous or arterial thrombotic events. Recently, the idea of the occurrence of thrombotic complications associated with liver transplant as a consequence of an uncontrolled activation of coagulation rather than of surgical complications has become more common. Usually, thrombotic events associated with LT can be divided into systemic thrombotic complications and regional vascular events (i.e., hepatic artery, portal vein, hepatic vein thrombosis). The incidence of early HAT, which may result in graft loss if arterial flow is not restored in the first 24 h after its occurrence, is approximately 3%-5% in adult patients, and the PVT incidence is approximately 2% in LT, with a very high mortality rate, ranging from 65% to 75%[34,35]. Hepatic vein thrombosis is instead considered a technical complication in case of a size mismatch of the grafts and in twisting and split liver transplant (i.e., reconstruction of the middle hepatic vein in extended right splits or insufficient drainage of the anterolateral sector in full-right grafts). Such dangerous complications can have surgical causes (for HAT: Difficult and prolonged arterial reconstruction, kinking of the artery, prolonged surgical time, prolonged cold or warmed ischemia times, the use of an aortic jump graft[36]; for PVT: Prior PVT or splenectomy, small portal vein size, use of venous conduits, insufficient portal flow due to large collaterals or systemic shunts[34]). Nevertheless, there are increasing data suggesting that changes in the hemostatic system (genetic factors, end-stage renal disease, diabetes, and history of prior DVT/PE) as well as intra- and postoperative blood products transfusion (increased transfusions of cryoprecipitate or fresh-frozen plasma and factor VII) may contribute to the development of HAT[37,38]. Interestingly Stine et al[39] have recently showed that pre-transplant PVT is associated with increased risk of early graft loss from HAT suggesting that hypercoagulability, besides surgical factors, could be involved in the post-transplant HAT occurrence[39].

DVT is a rare post-transplant complication that has a reported incidence between 3.5% and 8.6% in recent series[40]. Intraoperative systemic thrombotic complications, which mainly occur as acute PE or intracardiac right atrial thrombosis, are other dangerous events described in the perioperative period whose incidence of approximately 1%-4% seems to be higher than that reported in other surgical operations[41,42]. VTE is a well-documented postoperative complication, and PE is the most common cause of preventable death in surgical patients. Several risk factors for the development of PE during LT have been suggested, including vascular clamping, veno-venous bypass, central venous catheters, anti-fibrinolytic drugs, tissue injury/ischemia, venous stasis and etiology of liver disease.

In recent years, it has been shown that the cirrhotic patients are in a new hemostatic balance that undergoes new changes during liver transplant. During this surgical procedure, vWF remains elevated[43], and its functional capacity increases during surgery, probably due to an enhanced release of vWF from the activated endothelial cells. In association with that finding, the plasmatic concentration of ADMTS13-cleaving protease decreases during transplant, leading to an imbalance between vWF and ADMATS13, which is possibly responsible for the thrombotic risk.

Thrombocytopenia, which is typical of end-stage liver disease and potentially worsened by hemodilution and consumption during liver transplant, is not associated with a reduction in platelet function[44]. The preserved platelet adhesion, together with an imbalanced vWF/ADAMTS13 ratio, could somehow explain the increased incidence of thrombotic complications in the liver transplant perioperative period[45].

For secondary hemostasis, in cirrhotic patients, the parallel decline in pro- and anticoagulation proteins leads to a new balance characterized by normal thrombin generation. In the perioperative liver transplant period, the reduction of protein C, protein S, antithrombin III, and heparin cofactor II and the persistent increase in factor VIII lead to increased thrombin generation and to a hypercoagulable status[45]. During surgical procedures, the fibrinolytic system, which is normally in equilibrium in cirrhotic patients, can move temporarily towards hyperfibrinolysis in the anhepatic phase and after reperfusion[46]. However, at the end of surgery, a hypofibrinolytic condition, related to a massive increase in plasminogen activator inhibitor type 1 (PAI-1), develops and usually lasts up to 5 d after surgery[47]. Even if the reduction in the fibrinolytic activity can justify the occurrence of some thrombotic complication, such a causal relation has not been demonstrated by scientific works. The recent clinical literature seems to underline the role of the perioperative hypercoagulable status in the genesis of thrombotic complications such as HAT, PVT and other systemic thrombotic events.

Although perioperative bleeding complications occur more often than thrombotic ones, postoperative thromboses are associated with high morbidity and, in some cases, with mortality. Independent of the real origin of the prothrombotic status observed in cirrhotic patients who undergo LT, more efforts on the prevention of such complications by means of different prophylactic antithrombotic therapies are necessary. A prophylactic antithrombotic treatment to prevent these complications may be clinically relevant, and because of the fear of bleeding complications in the postoperative period, it could be useful to have an instrument that can reflect the coagulation status of the transplanted patient better than the routine laboratory tests can, which have been unreliable in reflecting the *in vivo* physiology.

HEMOSTATIC STATUS IN THE TRANSPLANTED PATIENT AND DIAGNOSTIC TOOLS

Standard coagulation tests
Standard coagulation tests have long been the standard laboratory indicators of patients' coagulation status. According to the cell-based model of hemostasis, these tests do not account for the important interactions between platelets, clotting factors, and other cellular components in the generation of thrombin or for the balance between coagulation and fibrinolysis. Furthermore, PT and PTT give only one piece of information, *i.e.*, whether the thrombin generation has started, but they give no information of what occurs afterwards.

Viscoelastic tests
Because of the limits of conventional coagulation tests in recognizing significant coagulopathies or guiding transfusion, in recent years the viscoelastic tests has gained increasing importance. Viscoelastic tests give an "in vitro" picture of the growth of the clot and a measure of its stability until its physiological or pathological lysis. These viscoelastic coagulation tests performed bedside, including ROTEM® and TEG®, produce faster and more reliable results than conventional tests do. These two commercial devices function by slightly different mechanisms. The thromboelastometer (ROTEM-analyzer, TEM International, Munich, Germany) uses a fixed cup with a pin that rotates, while the thrombelastographer (TEG-analyzer, Haemonetics Corp., Braintree, MA, United States) uses a fixed pin and a rotating cup[48,49].

TEG and rotational ROTEM are technologies that were previously applied in different surgical fields and are now applied to cirrhosis physiology. A more dynamic and targeted approach to the overall hemostatic process is at the basis of their success. They provide visual information on the coagulation process in terms

of maximal fibrin clot formation, fibrinolysis and tendency to hypercoagulability. These characteristics make these tests ideal for a rapid diagnosis of the type of coagulopathy and for an appropriate (and rational) choice of the therapeutic option in different fields, such as trauma care, cardiac surgery and LT[50]. Several studies have evaluated the effect of using TEG and ROTEM during LT on perioperative blood product transfusions, suggesting that a transfusion algorithm based on viscoelastic tests leads to reduced transfusions, but no survival benefit has been observed to date[51].

Actually, recent studies have noted that bleeding is no more the unique feared risk in liver cirrhosis. In the setting of liver surgery, several risk factors in addition to those previously described (vascular clamping, veno-venous bypass, central venous catheters, anti-fibrinolytic drugs, tissue injury/ischemia, venous stasis, etiology of liver disease, endothelial damages, ischemia time and vWF/ADAMT13 ratio imbalance) can increase the probability of thrombotic complications.

End-stage liver disease per se is a risk factor for VTE, and liver transplant surgery can improve the thrombotic condition. When dealing with cirrhotic patients, a test that could give information on hemorrhagic risk and the presence of normal clot stability or on the tendency to hypercoagulability would offer physicians an indication for prophylactic treatment without creating too much fear of the bleeding complications often associated with liver transplant. In the setting of liver disease and liver surgery, conventional coagulation tests give no or wrong information about hemostatic conditions, indicating hypocoagulability; in contrast, global viscoelastic tests show an enhanced hemostatic capacity or hypercoagulability.

TEG and ROTEM are seemingly superior tests of coagulation function in cirrhotic patients undergoing LT compared with traditional measures, but their capacity to recognize hypercoagulation status has yet to be demonstrated beyond much doubt.

In surgical settings, the study by Hincker and colleagues showed the encouraging results that ROTEM can predict thrombotic complications after major non-cardiac surgery[52]. Similarly, Kashuk and colleagues demonstrated that the presence of hypercoagulability identified by r-TEG is predictive of thromboembolic events in surgical patients[53]. When comparing TEG to INR in living-donor LT, TEG has been a useful tool to monitor for hypercoagulability in the perioperative period among liver donors, detecting a hypercoagulable state in some patients in spite of an elevated PT-INR[54].

Mallet *et al*[55] reported that several of the 124 liver recipients with end-stage liver disease presented with or developed a hypercoagulable thromboelastogram during liver transplant. Nevertheless, the 6 patients who developed early HAT had some TEG signs of hypercoagulability (increased cloth strength, high G,

or shortened reaction time), though it was not clear what kind of influence these alterations could have on thrombotic complication. The authors also showed that standard laboratory tests did not succeed in diagnosing any signs of hypercoagulability. Although hypercoagulability on a viscoelastic device can be associated with an increased risk of thromboembolic events in different surgical fields, trauma setting and critical care[53,56,57], the same association in cirrhotic patients seems more difficult to demonstrate. In these patients, the definition of hypercoagulability based on viscoelastic parameters is not unique, and it has included a shortening of reaction time (R time), an increase in maximal amplitude (MA), an increase in net clot strength (G), or a combination of these parameters. However, emerging evidence suggests that hypercoagulability detected by ROTEM or TEG can increase the probability of venous or arterial thrombotic complications in certain patients, such as those who undergo liver transplant. Lerner *et al*[58], in a review of 27 case reports of thromboembolic events during LT, showed that TEG profiles were hypercoagulable in more than 70% of cases. The study by Zanetto and colleagues showed that in cirrhotic patients with hepatocellular carcinoma, thromboelastometry could detect hypercoagulability, as identified by the presence of a shorter clotting time and higher maximum clot firmness, which was associated with PVT presence. In particular, an increased baseline MCF FIBTEM (> 25 mm) was associated with a 5-fold higher risk of developing PVT in cirrhotic Child A patients[59]. Recently, Zahr and colleagues analyzed the native procoagulant state of 828 LT recipients by using pre-transplant thromboelastographic data to identify risk factors for early HAT and found that the MA value was significantly higher in patients diagnosed with early HAT compared with those who were not. Specifically, an MA value on preoperative TEG of 65 mm or greater was recorded in a total of 7% of patients who went on to develop early HAT (hazard ratio = 5.28; 95% CI: 2.10-12.29; *P* < 0.001), whereas only 1.2% of patients with an MA less than 65 mm experienced this complication. The authors concluded that preoperative TEG may reliably identify group of recipients at greater risk of developing early HAT[60]. The use of viscoelastic tests in detecting any hypercoagulability condition during liver transplant seems helpful in better managing blood product transfusion or, hypothetically, prophylactic therapy, but it remains unclear whether these tests during the course of the surgery would be of additional outcome benefit, and several doubts remain about the reliability of their measures. Compared with the thrombin generation test (TGT), ROTEM and TEG may not be appropriate for hemostasis assessment in patients with liver cirrhosis, and it could lead to the unnecessary transfusion of fresh-frozen plasma or to the wrong administration of a thromboprophylactic drug. Even though Blasi *et al*[61]

reported that "parameters from the first derivative of the ROTEM are similar to the thrombin generation assay, which is considered the gold standard marker of hyper/hypocoagulability", several papers underline the lack of correlation between the ROTEM and TGT results. Lentschener and colleagues showed that while thromboelastometry detected a hypocoagulable profile in decompensated cirrhotic patients, TGT showed a normal to increased thrombin generation, suggesting a preserved hemostasis and/or a procoagulant state[62].

In addition to the non-uniformity of results between thromboelastography and TGT, non-uniformity has been recorded within an individual test (ROTEM or TEG). Some of the discrepancies can be partially explained by the differences in test methods, the activators used and the lack of viscoelastic test reference ranges for cirrhotic patients. The current habit to define hypo- or hypercoagulability by referring to reference ranges obtained from healthy individuals may not provide a reliable estimation of the coagulation status and bleeding risks of cirrhotics[63]. Other limitations of the use of ROTEM and TEG need to be considered when analyzing the information they give. Platelet dysfunction, either drug-induced or inherited, is not detected. These devices are insensitive to the effects of vWF and factor XIII[49,64,65], and they lack activation of the anticoagulant protein C system. Other shortcomings of TEG and ROTEM are the lack of adequate standardization, low reproducibility of the results and sensitivity to preanalytic variables[66,67].

Thrombin generation test

TGT is a promising laboratory tool for investigating hemorrhagic coagulopathies, predicting the risk of recurrent VTE after a first event, and monitoring patients on parenteral or oral anticoagulants[68]. In contrast to TEG and ROTEM, TGT can be performed with or without thrombomodulin, thus allowing us to analyze the natural anticoagulant protein C pathway. Furthermore, TGT offers more information on the hemostatic capacity in total because in contrast to viscoelastic tests, it assesses not only fibrin formation but the generation of thrombin, which does not stop when the fibrin clot has been generated[69]. For this reason, TGT best mimics the *in vivo* balance of pro- and anticoagulant proteins in plasma and the dynamics of thrombin generated *in vivo*[70].

TGT measures specific parameters such as the lag-time (time to start), the time to peak, the peak height, and the endogenous thrombin potential[69], so when used to analyze cirrhotic patients, it has shown a preserved or even increased thrombin generation, indicating a normal or even increased coagulation capacity, while ROTEM, performed in the absence of thrombomodulin, has shown hypocoagulation in proportion to the level of liver impairment[62].

Although TGT seems to better describe the inter-

actions between pro- and anticoagulant factors in the hemostatic process of patients with end-stage liver disease, this test is performed in platelet-poor plasma and/or platelet-rich plasma, which requires time to prepare and makes this method unsuitable for quick diagnosis and therefore impractical in a routine clinical setting.

Similar to TEG and ROTEM, this assay is not sufficiently standardized for broad clinical use, and the large variance of the preanalytic variables and the lack of standardized reference ranges prevent its routine clinical use[68]. Thrombin generation assays, although they are not readily available today, could provide a more effective tool for assessing the hemostatic system in patients with cirrhosis, but until further studies are performed, viscoelastic tests could be helpful for the clinical conditions associated with increased thrombotic risks by avoiding the overcorrection of the coagulation defects detected.

ROLE OF THROMBOPROPHYLAXIS

Review methodology

A systematic literature search was performed independently by two of the authors (LDP and RM) using PubMed, and the Cochrane Library Central. The search was limited to humans and articles reported in the English language. No restriction was set regarding the type of publication, date or publication status. Participants of adult age and any sex who underwent living transplantation or living donor liver transplantation procedures were considered. The search strategy was based on different combinations of words for each database. For the PubMed database, the following combination was used: ("liver transplantation" OR "liver transplant" OR "hepatic transplantation" OR "hepatic transplant") AND ("thromboprophylaxis" or "anticoagulation" or"antiplatelets" or "antithrombotic therapy" or "antithrombotic prophylaxis" or "prophylactic anticoagulation" or "anticoagulants" or "aspirin" or "heparin").

The same key words were inserted in the search manager fields of the Cochrane Library Central. The search was broadened by extensive cross-checking of reference lists of all retrieved articles fulfilling inclusion criteria. For all databases, the last search was run on March 28, 2018. The same two authors independently screened the title and abstract of the primary studies that were identified in the electronic search. The following inclusion criteria were set for inclusion in this systematic review: (1) studies reporting a thromboprophylactic therapy in liver transplant procedures; (2) studies reporting a description of the anticoagulation or antiplatelet therapy performed in liver transplant recipients; and (3) if more than one study was reported by the same institute, only the most recent or the highest quality study was included.

The following exclusion criteria were set: (1) letters, comments and case reports; and (2) studies where it was impossible to retrieve or calculate data of interest.

The same two authors extracted the following main data: (1) first author, year of publication and study type; (2) number and characteristics of patients; (3) effectiveness of the thromboprophylaxis performed in term of portal vein thrombosis, hepatic artery thrombosis, deep vein thrombosis and pulmonary embolism; and (4) complications of thromboprophylaxis. Bias of the individual studies was categorized based on study design. All relevant texts, tables and figures were reviewed for data extraction. Discrepancies between the two reviewers were resolved by consensus discussion.

The literature search yielded 634 articles; after the removal of all the articles that did not reflect the inclusion and exclusion criteria, a total of 11 articles[62,71-79] published between 1997 and 2018 were included in this systematic review. Three studies were prospective[71,76,80], only one was a prospective case control study[75] while all the others were retrospective[61,72,74] and four of these had control group[73,77-79]; No papers reported multicentric data. All these studies included a total of 5192 patients (adult and children).

Description of the studies

Despite the altered coagulation tests and thrombocytopenia, patients with end-stage liver disease are at a risk for thrombosis[28]. Several authors suggested that routine thromboprophylaxis should therefore not be withheld from hospitalized patients with liver disease[81] unless risk factors for bleeding are present. Recent advances in the understanding of the coagulopathy in chronic liver disease have provided strong support for anticoagulation as a new therapeutic paradigm for patients with cirrhosis, which would be able to decrease the progression of the liver disease[81].

Although the incidence of venous thrombosis after LT is similar to that reported in other types of major surgery, the fact that these complications occur despite hypocoagulable routine laboratory tests in the first postoperative days indicates that these routine tests probably do not reflect the *in vivo* physiology. The lack of reliable laboratory tests and the contraindications to thromboprophylaxis in cases of high hemorrhagic risk, such as that recognized in LT, make the management of anticoagulants complex in this surgical setting. After LT, patients may be hypercoagulable because of an imbalance between coagulation and fibrinolytic mechanisms, tipping towards a prothrombotic state in the early postoperative phase[82]. It has been established as well that after LT and partial hepatectomy, patients are hypercoagulable because of enhanced thrombin generating capacity, despite prolongations in the PT[83-85]. Furthermore, postoperative immunosuppressive drugs may play a role in increasing platelet aggregation and thrombogenicity[86]. However, usually physicians find

several difficulties in administering thromboprophylaxis because they do not know if, in the postoperative period, the coagulopathy persists or the coagulation balance reverts to normality. In the light of the above mentioned observation thromboprophylaxis should not be withheld on basis of post-operative prolonged PT values, as in reality coagulation is hyperactive and standard coagulation test are not truly representative of the real coagulation status in these patients. Other difficulties come from the lack of effective predictors of VTE. INR, MELD score and platelet number have been unreliable to predict thromboembolic complications[25]. Due to the risks of bleeding and coagulopathy in the postoperative LT period, antithrombotic prophylaxis to prevent VTE is not routinely used, and no consensus exists.

Recently, Mukerji and colleagues advised delaying anticoagulation until the post-transplant INR was above 1.5 to 2.0 and the platelet count was below 50000[9]. Similarly, Blasi and colleagues suggested administering thromboprophylaxis with low-molecular-weight heparin to patients with Child A cirrhosis and in patients who undergo intraoperative thrombectomy, avoiding its administration if the platelet count is under 30×10^9/L or in cases of significant intraoperative blood loss[61].

Even if thromboprophylaxis in the early postoperative period of cadaveric liver transplant recipients is not routinely administered and the reports on the usage of heparin are very limited, more surgeons have begun to implement thromboprophylaxis therapy to reduce the risk of vessel thrombosis (Table 2). Most liver transplant centers have developed their own protocols for heparin infusion and the monitoring of its activity, even though bleeding remains the most feared complication associated with anticoagulation therapy, especially in cases of delayed graft function and marginal graft. For instance, Kaneko *et al*[71] reported a high incidence (9%) of surgical revision for hemorrhagic complications in their living related liver recipients who received unfractionated heparin (UFH). They suggested that the dose of heparin should be adjusted to maintain activated clotting time (ACT) levels lower than the previously settled ones during the early postoperative period. Mori *et al*[80] declared that their basic protocol after living donor LT for the patients who underwent portal reconstruction for PVT did not include anticoagulation therapy. Only patients with good coagulation (PT-INR < 1.5) or slow portal flow were administered intravenous heparin at the dose of 5 U/kg/h during the first week after the LT and only then shifted to warfarin[80]. Similarly, Stange and colleagues suggested to administer low-dose heparin as a continuous infusion of 5.000 IE over 24 h, beginning 6 h postoperatively, for 14 d only in case of split-liver transplantation or complex arterial reconstruction[72].

In contrast to Kaneko and Mori, Yip and colleagues[73] implemented a standardized prophylactic regimen in LT recipients with subcutaneous heparin

Table 2 Different thromboprophylaxis protocols for postoperative arterial and venous thrombosis in liver transplant patients

Authors	Type of study	Drug used	Target population	Number Pts	PVT	HAT	Bleeding	Observations
Blasi 2016[61]	Retrospective study No controls	Enoxaparin not routinely, unless intraoperative. thrombectomy or the patient was under anticoagulant treatment before LT. No thromboprophylaxis if the platelets are under 30×10^9/L.	Adult LT	328	8 (2.4%)	NA	Not reported	5/8 patients with PVT did not receive prophylaxis, and the other 3 received it days after LT or in only a few doses
Kaneko 2005[71]	Prospective study No controls	Dalteparin administration adjusted with reference to the ACT (130-160 s)	Adult Living-donor LT	128	1 PVT (0.78%) and 1 (0.78%) PVT + HAT	2 HAT (1.5%) and 1 HAT + PVT (0.78%)	11 (8.5%) surgical revisions and 8 (6.25%) patients with hemorrhages complications treated conservatively	High hemorrhage complication rate in this series indicates that a lower target ACT range may be preferable in the second post-operative week.
Gad 2016[74]	Retrospective study No controls	Heparin infusion up to 180-200 units/kg/day adjusted with reference to the ACT (target levels, 180-200 s) and/or the aPTT (target levels, 50-70 s).	Adult and pediatric living-donor LT	186	5 (2.3%)	4 HAT (1.8%) 4 HAT and PVT (1.8%)	4 (1.8%)	Pre-LT PVT may deserve more intensive anticoagulation therapy
Sugawara 2002[76]	Prospective study No Controls	LMWH, ATIII, prostaglandin E1 (0.01 g/kg/h) and a protease inhibitor	Adult Living-donor LT	172	4 (2.3%) both PVT + HAT	7 (4.0%)	Not considered	The authors' strategy against HAT is aimed to correct the imbalance between the coagulation and anticoagulation systems
Mori 2017[80]	Prospective study No controls	Heparin infusion at the dose of 5 U/kg/h during the first week after LT	Adult Living-donor LT	282 total patients; 48 patients with pre-existing PVT; number of patient with thromboprophylaxis not cited	8 (17%)	NA	Not considered	The basic protocol after LDLT does not provide anticoagulant therapy. Only patients with good coagulation (INR) < 1.5 or slow portal flow (velocity < 10 cm/s) and intraoperative portal reconstruction for PVT were administered intravenous heparin. The aim of this study was to determine the risk factors that influence the incidence of DVT/PE and the effectiveness of prophylaxis
Yip 2016[73]	Retrospective case control study	Subcutaneous heparin (5000 U) every 8 h	Adult LT	999 total patients; 288 patients with thromboprophylaxis from 2011	Not considered	Not considered	NA	The aim of this study was to determine the risk factors that influence the incidence of DVT/PE and the effectiveness of prophylaxis

Uchikawa 2009[75]	Prospective case control study	Continuous i.v. Dalteparin infusion administered in the anhepatic phase to maintain the ACT levels from 140 to 150 seconds (Gr.A) vs continuous i.v Dalteparin infusion administered immediately after the operation and adjusted depending on clinical findings (Gr.B)	Adult Living-donor LT	42 total patients (10 vs 32)	0 % in Gr. B 5 (15.6%) in Gr. A	0 % in Gr. B 5 (15.6%) in Gr. A	0% in Gr. B 1 (3.1%) in Gr. A	The study evaluated the advantage of ACT as a reliable tool for bedside monitoring of LMWH anticoagulant effects during and following LDLT
Stange 2003[72]	Retrospective study No controls	UFH 5000 IU over 24 h beginning 6 h postoperatively, for 14 d	Adult Living-donor LT	1192	Not evaluated	14 (1.17%)	3 (0.2%) bleeding episodes not apparently related to UFH	The authors analyzed the incidence, clinical presentation, therapeutic options, and outcome of hepatic artery thrombosis (HAT)
Wolf DC, 1997[77]	Retrospective case control study	81 mg oral aspirin in adult and 40 mg in children from postoperative day 1	Adult and pediatric LT	499 total patients (354 vs 175)	Not evaluated	10 (2.9%) vs 6 (3.6%) in the not treated group	89 (16.8%) gastrointestinal bleeding 66 treated vs 23 not treated patients	The spontaneous or invasive maneuver-related bleeding episodes were more frequent in the treated group
Vivarelli 2007[78]	Retrospective case control study	100 mg aspirin	Adult LT	838 total patients (236 treated vs 592 not treated)	Not evaluated	1/236 (0.4%) treated patients vs 13/592 (2.2%) not treated patients	0%	The aim of this study was to determine the safety and efficacy of aspirin therapy on late HAT
Shay 2013[79]	Retrospective case control study	325 mg aspirin	Adult LT	469 total patients (165 treated vs 304 not treated)	6/304 (2%) not treated patients vs 1/165 patients treated (0.6%)	15/304 patients (4.9%) vs 5/165 (3%) patients overall HAT (early and late) in the control and treated group	Similar bleeding rates between the two groups	The aim of this study was to determine the safety and efficacy of early aspirin therapy on clinical outcomes

LT: Liver transplant; PVT: Portal vein thrombosis; HAT: Hepatic artery thrombosis; ACT: Activated clotting time; aPTT: Activated partial thromboplastin time; DVT: Deep vein thrombosis; PE: Pulmonary embolism; LMWH: Low-molecular-weight heparin; UFH: Unfractionated heparin.

(5000 U) every 8 h, demonstrating that this therapy significantly reduces VTE events without increasing bleeding risks. In support of this kind of thromboprophylaxis, other studies have found that unfractionated heparin did not increase the risk of bleeding in patients with cirrhosis[87,88]. Gad and colleagues[74], in a retrospective work on 222 adult and pediatric living-donor LTs, reported their standard prophylactic therapy (heparin infusion up to 180-200 units/kg per day adjusted with reference to the ACT and/or the aPTT) and concluded that a more intensive anticoagulation therapy could be one option, especially when dealing with preoperative PVT. Uchikawa et al[75], fearing bleeding complications associated with UFH administration, proposed a thromboprophylactic regimen with dalteparin, a relatively selective inhibitor of factor Xa activity. In their prospective case-control group, they showed that anticoagulation therapy, based on ACT, reduces thrombotic complications without increasing bleeding[75]. Similarly, Sugawara et al[76] demonstrated the efficacy of intensive anticoagulation obtained with low-molecular-weight heparin (LMWH) administration.

In addition to LMWH and UFH, antiplatelet drugs have been proposed for prophylaxis after LT because of the important role of platelets in thrombotic complication. Wolf and colleagues in 1997 showed no benefit of prophylactic low-dose aspirin therapy in the prevention of early HAT after liver transplantation[77]. Differently, Vivarelli et al[78], in a single-center retrospective study, examined the effect of long-term aspirin administration (100 mg) on the incidence of late HAT in a large number of patients. They found a relative risk reduction of 82% without any recorded bleeding episodes throughout the follow-up period. Unfortunately, one of the major limitations of the

Table 3 Possible conditions warranting thromboprophylaxis in the postoperative period of liver transplant recipients
Living-donor liver transplant and split liver
Surgical difficulties and complex vessel reconstruction
Presurgical portal vein thrombosis
Intraoperative portal or hepatic artery thrombectomy
Hypoplastic portal vein
Jump graft artery reconstruction
Cholestatic recipient diseases and Budd-Chiari disease
Non-alcoholic fatty liver disease/non-alcoholic steatohepatitis
Small or multiple recipient arteries
Low portal or arterial blood flow intraoperatively

study was the inability to verify the effect of aspirin on early HAT because of authors' inability to start aspirin immediately after LT in all patients with known impaired coagulative function or with a high risk of bleeding[78]. In contrast to Vivarelli, Shay *et al*[79] showed that aspirin prophylaxis is safe and effective in decreasing early HAT in adult recipients, making the HAT incidence decrease from 3.6% to 0% in the treated group. There was no difference in bleeding complications between the groups, regardless of the dosage of aspirin (325 mg) used.

There are emerging data on the safety of anti-coagulants in patients with cirrhosis[81,89]. Several groups recommend thromboprophylaxis in the absence of clear contraindications in the clinical, but not surgical, setting[87,90]. A different situation is represented by LT, where guidelines for VTE prophylaxis are lacking from both safety and efficacy standpoints. In this setting, a careful risk stratification, a wise drug choice and a reliable monitoring of drug effects may orient the choice on whether cirrhotic patients could benefit from prophylaxis (Table 3).

DRUG CHARACTERISTICS AND MONITORING ASSAY

In general, liver transplant surgery guidelines regarding VTE prophylaxis are lacking from both safety and efficacy standpoints. However, consideration should be given to using both mechanical and chemical prophylaxis after LT. Some studies, in fact, have reported rates as high as 79.6% of transplants being undertaken without transfusion[91] and a risk of developing a DVT after LT with mechanical prophylaxis alone ≥ 9%[40]. The growing evidence from clinical studies of the normal or even increased coagulation status in patients with cirrhosis, which has replaced the old dogma of the "auto-anticoagulated patient", has oriented most physicians to a more frequent postoperative thromboprophylaxis administration. Therefore, several warnings must be considered before implementing a more liberal use of anticoagulants in patients who have undergone liver transplant, and the risks and benefits of anticoagulation in these patients must be carefully

weighed. Some of these warnings regard the chosen drug. Unfractionated heparin and LMWH are the most frequently used drugs, although there are several concerns and a lack of consensus on the required doses, the efficacy of treatment and the best way to monitor the pharmacologic effects achieved.

Unfractionated heparin
Heparin is used to reduce the incidence of HAT after liver transplantation. The anti-coagulatory effect of UFH comes mainly from its capacity to enhance the endogenous anticoagulant antithrombin III activity, from its effects on platelets, platelet factor 4, the fibrinolytic system and thrombin[92,93]. In liver cirrhosis or liver failure, the hepatic synthesis of coagulation factors, including antithrombin, is impaired, affecting anti-Xa testing and the predictability of UFH's anticoagulation effect[94]. In cirrhotic patients, a discrepancy has been reported between the anti-Xa level and the activated partial thromboplastin time while monitoring UFH therapy[95]. The anti-Xa tests underestimate the UFH levels, whereas the aPTT gives an overestimation[96]. Neither of these tests assesses drug levels directly; rather, they estimate the drug levels starting from the anticoagulant action of the drug.

When monitoring unfractionated heparin's effects by aPTT modifications, physicians must address an already prolonged aPTT value in many patients with cirrhosis, making the aPTT target ranges for these patients unclear. The aPTT test has not been assessed in cirrhotic patients, and its targeted range is unclear given that aPTT is prolonged at baseline in patients with cirrhosis. In this case, the level of ant-Xa is usually decreased, and that of aPTT is increased. This condition brings into question whether the augmentation of UFH doses based on anti-Xa level could expose patients to a higher risk of bleeding or if decreasing the UFH dose based on aPTT values could predispose patients to subtherapeutic therapy.

The fluctuating liver graft synthetic capacity and the consequently variable antithrombin level in the plasma of the transplanted patients make the heparin anticoagulation activity extremely variable during the postoperative period, requiring continuous monitoring and dose adjustment. Thus, understandable fears of bleeding are common in the post-liver transplant period.

Hemorrhagic complication can occur with the poorly monitored use of heparin. Kaneko *et al*[71] reported that 9% of their living related liver recipients who used UFH developed hemorrhagic complications that required surgical treatment. Other authors[74] noted the safety profile of heparin infusion in a liver transplant setting, underlining the necessity to obtain several samples to monitor its activity.

The ACT monitors the activity of the intrinsic pathway in the coagulation system and has been used after liver transplant to assess heparin anticoagulation.

Different target levels, depending on the clinical study, have been described and range from 130-160 s[71] and 180-200 s[74]. Most liver transplant centers have developed their own protocols for heparin infusions and heparin activity monitoring based on empirical rules. Because of the lack of consensus on the target ranges to be reached to obtain anticoagulation without increasing the hemorrhagic risk too much, authors have chosen the target limit arbitrarily, and surprisingly, the same target range has been both associated and not associated with bleeding complications in different clinical papers.

Another limit of UFH administration is heparin-induced thrombocytopenia (HIT), which is an adverse immune-mediated reaction to heparin that results in platelet count decreases of more than 50% within 5 to 10 d after heparin administration. The prevalence of HIT or HIT antibody in the liver recipient population seems to be very low[97], with the exception of Budd-Chiari syndrome patients, in whom the HIT prevalence is significantly higher than the general population[98]. Nevertheless, the careful monitoring of platelet count and possible thrombosis is necessary when heparin therapy is prolonged or patients have a history of heparin therapy.

Despite these limitations, UFH pharmacokinetics, especially if administered at low doses, characterized by a rapid short-term action, poor bioavailability, and a rapidly reversible anticoagulant effect in case of hemorrhagic complications, makes UFH a good anticoagulant in liver transplant patients with concomitant renal failure. Because of the limitations of both anti-Xa and aPTT tests in estimating the drug levels in plasma, thrombin generation testing could represent a valid alternative that offers information about the true anticoagulant effect of this drug; unfortunately, this test is not available for routine clinical use[96].

Low-molecular-weight heparin

LMWH selectively inhibits clotting factor X and, augments antithrombin III activity[71]. Despite being considered a drug with less risk of bleeding[99] than unfractionated heparin due to its selective inhibition of coagulation factor X, and because of its reduced ability to bind to platelet factor 4 and von Willebrand factor, its anticoagulation activity may be difficult to predict[99]. The anticoagulation efficacy in the presence of both graft and renal dysfunction may be extremely variable, and a reduction in the dosage of LMWH is recommended because it has increased anticoagulant potency in patients with cirrhosis[100]. Hence, in patients with impaired renal function, which is commonly seen following LT, monitoring and dose adjustments according to the degree of renal injury are required. For this reason, in patients with a high bleeding tendency, such as liver transplant recipients, an adjustable continuous infusion of LMWH may be recommended

to avoid peak plasma levels and to better manage the continuous changes in the coagulation cascade related to the graft functionality[75].

While LMWH reduces ischemia-reperfusion-associated liver damage, which may make it more advantageous than UFH for intraoperative and postoperative anticoagulant therapy in LT, continuous monitoring and dose adjustments according to the degree of renal function are required. The most widely used test that correlates with the administered LMWH dose is the anti-FXa activity in plasma, which measures the inhibitory activity of LMWH-antithrombin (AT) complexes towards FXa. However, the assay is prone to several pitfalls that need to be considered when it is used to monitor the cirrhotic patient[101]. The low levels of antithrombin typical of cirrhotic patients limit the formation of LMWH-AT-FXa complexes and lower the ability of the anti-FXa activity assay to guide LMWH dosage[96]. Potze and colleagues found that the anti-FXa assay underestimated the LMWH dose administered in vitro to plasma of cirrhotic patients and had dangerous clinical consequences[96]. The low reliability of the anti-FXA assay, which underlines a persistently low anti-FXA activity, can lead to dangerous LMWH dose escalation in the postoperative period in liver transplant recipients[102]. To overcome this limitation of the assay at low AT levels, some authors propose the addition of AT before monitoring LMWH, which is not the standard practice in most routine diagnostic laboratories[81]. Another alternative could be represented by the ACT, which is the most commonly used and sensitive monitoring method to verify UFH's effects. Some reports have documented that ACT is not reliable in monitoring LMWH's effect[103,104], and the ACT cannot usually monitor the activity of factor Xa. However, in other clinical papers, ACT did monitor the anticoagulatory effect of LMWH in coronary intervention procedures[105] and in living-donor LT[75]. Uchikawa *et al*[75] showed that ACT measurement is a simple, reliable method for bedside monitoring of LMWH's (dalteparin's) anticoagulant effects for living-donor LT.

The usefulness of the conventional ACT for LMWH monitoring is still under debate. TGT could represent a valid alternative[106]. However, this test is still impractical in a routine clinical setting, as it requires time to prepare and is not suitable for quick diagnosis.

Antiplatelet

The constant activation of platelets in the perioperative period of liver transplant, which can lead to platelet consumption and sequestration in the liver following graft reperfusion, has been associated with the occurrence of arterial thrombosis and graft failure[44]. Because of the significant role of platelets in the development of thrombosis, antiplatelet therapy has come to be seen as an attractive preventive therapy. Aspirin's ability to suppress the production of prostaglandins and thromboxanes is due to its irreversible inactivation of

the cyclooxygenase (COX) enzyme, which leads to an interference with platelet aggregation and to an endothelial cell-mediated inhibition of the coagulation cascade[107]. Although the widespread use of aspirin seems attractive, the traditional fear of hemorrhagic complication in the postoperative period of the liver transplant patient is responsible for the general avoidance of anticoagulant medication in these patients and for the very few studies performed on the efficacy and safety of antiplatelet drugs after liver transplantation. The literature on antiplatelet administration in the post-transplant period is controversial. Wolf and colleagues (1997) found no benefit of prophylactic low-dose aspirin therapy in the prevention of early HAT after liver transplantation, and even if aspirin administration did not seem to be associated with postoperative bleeding complications, a trend toward an increased incidence of gastrointestinal bleeding was seen[77]. In contrast, Vivarelli *et al*[78]. reported the efficacy and safety of long-term aspirin administration for the occurrence of late HAT, without hemorrhagic complications associated with aspirin administration. Similarly, Shay *et al*[79], who used a higher dosage of aspirin immediately after surgery, showed that aspirin prophylaxis was safe and effective in decreasing early HAT in adult recipients, with no evidence of significant bleeding. These recent works seem to suggest that thromboprophylactic anticoagulation with aspirin in selected high-risk LT patients may be considered carefully.

Antiplatelet agents, in contrast to other anti-coagulants, could offer the advantage of not requiring laboratory monitoring for dose adjustments. No tests are usually performed to avoid excessive inhibition of platelet function, and the issues regarding monitoring in the use of other anticoagulants cannot be applied to antiplatelet drugs. Unfortunately, laboratory tests of platelet function are frequently abnormal in patients with cirrhosis, and it may be challenging to detect the efficacy of anti-platelet agents in a category of patients with thrombocytopenia and platelet function alterations[108]. Furthermore, despite the clinical evidence for the efficacy of aspirin for early and late HAT, the use of aspirin has been associated with an increased risk of a first variceal bleeding in patients with established varices[109] and with acute renal failure, hyponatremia, and diuretic resistance in patients with ascites[110], occasionally making preventive aspirin administration in liver recipients difficult.

Clopidogrel

P2Y12 blockers such as clopidogrel, which has an active metabolite that irreversibly inhibits the ADP P2Y12 receptor, have been proposed to prevent post-transplant arterial thrombosis, but the risk of thrombosis vs bleeding must be wisely considered when choosing this drug in the postoperative period[7].

Perioperative management of antiplatelet therapy in patients who undergo surgery with a high hemorrhagic risk is difficult and should be formulated by a team of experts (surgeon, anesthesiologist), who should weigh the relative risk of bleeding with that of thrombosis. The risk of bleeding complications in the perioperative period has been increased 1.5-fold under low-dose aspirin[111], and when clopidogrel is not discharged within 7 d prior to the operation, this risk increases to 30%[112]. Clopidogrel is correlated with increased bleeding and is usually interrupted in the postoperative period until the risk has been reduced. Clopidogrel's hemorrhagic risk, the lack of established reversal agents, the fact that it is widely metabolized by the liver and excreted in urine, and the risk of excessive anticoagulation in the immediate postoperative period have made this drug barely manageable and, thus, not indicated for postoperative antithrombotic prophylaxis.

Direct oral anticoagulant agents

Direct oral anticoagulant agents (DOACs) have been proposed as an attractive alternative to heparin and LMWH for the prevention of post-transplant thrombotic complications. Moreover, preliminary data show that the use of DOAC is safe in cirrhotics[113]. These drugs have the advantages of oral administration, fixed dose and no need for laboratory monitoring. Moreover, their mechanism of action is independent of antithrombin, which is necessary for LMWH be effective but may be severely impaired in cirrhosis patients. However, we know of no DOAC study in postoperative liver transplant patients. The experience with DOACs is still limited, their anticoagulation effect is not quickly reversible, their elimination route is through the kidney and liver, and excessive drug accumulation could be associated with bleeding issues in the postoperative period. In liver transplant recipients, renal and liver function are often impaired, and excessive anticoagulation effects could develop, making DOACs of scarce interest for postoperative thromboprophylaxis.

CONCLUSION

The widespread fear of postoperative bleeding after LT has been partly reduced by the awareness of the real coagulation balance of cirrhotic patients and by the increasing number of transplants performed without the need for transfusion. The old concept of the cirrhotic patient as an anticoagulated patient and the idea that the normal coagulation tests are able to represent the real coagulation balance of the patient have now been replaced. With this new knowledge, the attention towards thromboembolic complications of the liver transplant patients has become increasingly pressing. An unbalanced coagulation system toward hypercoagulability may persist for a variable period of time after the liver transplant. The delayed recovery of the anticoagulant factors and the reaching of normal activity among almost all of the procoagulant proteins not before day 1 to 3 postoperatively have been

widely described[82]. The increasing awareness that hypercoagulability can represent a serious risk in the perioperative period of the liver recipient, together with the lack of reliable tests to verify the presence of this condition, should increase the awareness of physicians of the need for adequate thromboprophylaxis therapy. Literature on this matter is scarce, and the degree of anticoagulation to be achieved or the tests to monitor anti-coagulation are not the result of common consensus. Thromboprophylaxis should be used more often. From the literature available, we infer that the most commonly used drugs for antithrombotic purposes in patients undergoing LT are UFH, LMWH and cardioaspirin. The use of DOAC and clopidogrel in this category of patients is still limited, due in particular to their slowly reversible effect and the excessive anticoagulation effect they can cause because of their elimination route. Likewise, the possibility of hemorrhagic complications in transplanted patients remains a real fear. Pharmacological prophylaxis is probably beneficial in reducing the incidence of thromboembolic complications in the perioperative period, but a careful patient selection and a reliable coagulation test to monitor the pharmacological prophylaxis are needed. Thrombin-generation assays are the most reliable tests to represent the net amount of thrombin that can be generated as a result of the pro- and anticoagulant drivers and is a promising laboratory tool for investigating hemorrhagic and thrombotic coagulopathies. However, this test is still impractical in a routine clinical setting because it requires time to prepare and is not suitable for quick diagnosis. While waiting for this test to be readily available for bedside testing, viscoelastic tests, with all their limitations, could be helpful for the clinical conditions associated with increased thrombotic risks by avoiding the overcorrection of coagulation defects.

Unfortunately, because of the lack of a coagulation test that reliably predicts the risk of bleeding or thrombosis in the perioperative LT period, strong recommendations on thromboprophylaxis in this setting cannot be made, in particular in patients with delayed graft function. More well-designed clinical studies on the efficacy, safety, and tolerability of anticoagulant drugs for the prevention of thrombotic events in transplanted patients are needed.

REFERENCES

1   **Lisman T**, Caldwell SH, Burroughs AK, Northup PG, Senzolo M, Stravitz RT, Tripodi A, Trotter JF, Valla DC, Porte RJ; Coagulation in Liver Disease Study Group. Hemostasis and thrombosis in patients with liver disease: the ups and downs. *J Hepatol* 2010; **53**: 362-371 [PMID: 20546962 DOI: 10.1016/j.jhep.2010.01.042]

2   **Runyon BA**; AASLD. Introduction to the revised American Association for the Study of Liver Diseases Practice Guideline management of adult patients with ascites due to cirrhosis 2012. *Hepatology* 2013; **57**: 1651-1653 [PMID: 23463403 DOI: 10.1002/hep.26359]

3   **Lisman T**, Porte RJ. Rebalanced hemostasis in patients with liver disease: evidence and clinical consequences. *Blood* 2010; **116**: 878-885 [PMID: 20400681 DOI: 10.1182/blood-2010-02-261891]

4   **Liang TB**, Bai XL, Li DL, Li JJ, Zheng SS. Early postoperative hemorrhage requiring urgent surgical reintervention after orthotopic liver transplantation. *Transplant Proc* 2007; **39**: 1549-1553 [PMID: 17580186 DOI: 10.1016/j.transproceed.2007.01.080]

5   **Hendriks HG**, van der Meer J, de Wolf JT, Peeters PM, Porte RJ, de Jong K, Lip H, Post WJ, Slooff MJ. Intraoperative blood transfusion requirement is the main determinant of early surgical re-intervention after orthotopic liver transplantation. *Transpl Int* 2005; **17**: 673-679 [PMID: 15717214 DOI: 10.1007/s00147-004-0793-5]

6   **Ishitani M**, Angle J, Bickston S, Caldwell S, Isaacs R, Pruett T. Liver transplantation: incidence and management of deep venous thrombosis and pulmonary emboli. *Transplant Proc* 1997; **29**: 2861-2863 [PMID: 9365593]

7   **Feltracco P**, Barbieri S, Cillo U, Zanus G, Senzolo M, Ori C. Perioperative thrombotic complications in liver transplantation. *World J Gastroenterol* 2015; **21**: 8004-8013 [PMID: 26185371 DOI: 10.3748/wjg.v21.i26.8004]

8   **Qi X**, Dai J, Jia J, Ren W, Yang M, Li H, Fan D, Guo X. Association between portal vein thrombosis and survival of liver transplant recipients: a systematic review and meta-analysis of observational studies. *J Gastrointestin Liver Dis* 2015; **24**: 51-59, 4 p following 59 [PMID: 25822434 DOI: 10.15403/jgld.2014.1121.qix]

9   **Mukerji AN**, Karachristos A, Maloo M, Johnson D, Jain A. Do postliver transplant patients need thromboprophylactic anticoagulation? *Clin Appl Thromb Hemost* 2014; **20**: 673-677 [PMID: 24917126 DOI: 10.1177/1076029614538490]

10   **Guyatt GH**, Akl EA, Crowther M, Gutterman DD, Schünemann HJ; American College of Chest Physicians Antithrombotic Therapy and Prevention of Thrombosis Panel. Executive summary: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest* 2012; **141**: 7S-47S [PMID: 22315257 DOI: 10.1378/chest.1412S3]

11   **Tripodi A**. Hemostasis abnormalities in cirrhosis. *Curr Opin Hematol* 2015; **22**: 406-412 [PMID: 26203733 DOI: 10.1097/MOH.0000000000000164]

12   **Violi F**, Basili S, Raparelli V, Chowdary P, Gatt A, Burroughs AK. Patients with liver cirrhosis suffer from primary haemostatic defects? Fact or fiction? *J Hepatol* 2011; **55**: 1415-1427 [PMID: 21718668 DOI: 10.1016/j.jhep.2011.06.008]

13   **Lisman T**, Bongers TN, Adelmeijer J, Janssen HL, de Maat MP, de Groot PG, Leebeek FW. Elevated levels of von Willebrand Factor in cirrhosis support platelet adhesion despite reduced functional capacity. *Hepatology* 2006; **44**: 53-61 [PMID: 16799972 DOI: 10.1002/hep.21231]

14   **Tripodi A**, Primignani M, Chantarangkul V, Clerici M, Dell'Era A, Fabris F, Salerno F, Mannucci PM. Thrombin generation in patients with cirrhosis: the role of platelets. *Hepatology* 2006; **44**: 440-445 [PMID: 16871542 DOI: 10.1002/hep.21266]

15   **Leebeek FW**, Rijken DC. The Fibrinolytic Status in Liver Diseases. *Semin Thromb Hemost* 2015; **41**: 474-480 [PMID: 26049070 DOI: 10.1055/s-0035-1550437]

16   **Caldwell SH**, Hoffman M, Lisman T, Macik BG, Northup PG, Reddy KR, Tripodi A, Sanyal AJ; Coagulation in Liver Disease Group. Coagulation disorders and hemostasis in liver disease: pathophysiology and critical assessment of current management. *Hepatology* 2006; **44**: 1039-1046 [PMID: 17006940 DOI: 10.1002/hep.21303]

17   **Colucci M**, Binetti BM, Branca MG, Clerici C, Morelli A, Semeraro N, Gresele P. Deficiency of thrombin activatable fibrinolysis inhibitor in cirrhosis is associated with increased plasma fibrinolysis. *Hepatology* 2003; **38**: 230-237 [PMID: 12830006 DOI: 10.1053/jhep.2003.50277]

18   **Levi M**, van der Poll T. Coagulation and sepsis. *Thromb Res* 2017; **149**: 38-44 [PMID: 27886531 DOI: 10.1016/j.thromres.2016.11.007]

19   **Senzolo M**, Agarwal S, Zappoli P, Vibhakorn S, Mallett S, Burroughs AK. Heparin-like effect contributes to the coagulopathy in patients with acute liver failure undergoing liver transplantation.

Liver Int 2009; **29**: 754-759 [PMID: 19220741 DOI: 10.1111/j.1478-3231.2009.01977.x]

20   **Tripodi A**, Caldwell SH, Hoffman M, Trotter JF, Sanyal AJ. Review article: the prothrombin time test as a measure of bleeding risk and prognosis in liver disease. *Aliment Pharmacol Ther* 2007; **26**: 141-148 [PMID: 17593061 DOI: 10.1111/j.1365-2036.2007.03369.x]

21   **de Franchis R**; Baveno VI Faculty. Expanding consensus in portal hypertension: Report of the Baveno VI Consensus Workshop: Stratifying risk and individualizing care for portal hypertension. *J Hepatol* 2015; **63**: 743-752 [PMID: 26047908 DOI: 10.1016/j.jhep.2015.05.022]

22   **Haas T**, Fries D, Tanaka KA, Asmis L, Curry NS, Schöchl H. Usefulness of standard plasma coagulation tests in the management of perioperative coagulopathic bleeding: is there any evidence? *Br J Anaesth* 2015; **114**: 217-224 [PMID: 25204698 DOI: 10.1093/bja/aeu303]

23   **Lisman T**, Leebeek FW, Mosnier LO, Bouma BN, Meijers JC, Janssen HL, Nieuwenhuis HK, De Groot PG. Thrombin-activatable fibrinolysis inhibitor deficiency in cirrhosis is not associated with increased plasma fibrinolysis. *Gastroenterology* 2001; **121**: 131-139 [PMID: 11438502]

24   **Hugenholtz GC**, Macrae F, Adelmeijer J, Dulfer S, Porte RJ, Lisman T, Ariëns RA. Procoagulant changes in fibrin clot structure in patients with cirrhosis are associated with oxidative modifications of fibrinogen. *J Thromb Haemost* 2016; **14**: 1054-1066 [PMID: 26833718 DOI: 10.1111/jth.13278]

25   **Northup PG**, McMahon MM, Ruhl AP, Altschuler SE, Volk-Bednarz A, Caldwell SH, Berg CL. Coagulopathy does not fully protect hospitalized cirrhosis patients from peripheral venous thromboembolism. *Am J Gastroenterol* 2006; **101**: 1524-8; quiz 1680 [PMID: 16863556 DOI: 10.1111/j.1572-0241.2006.00588.x]

26   **Vukovich T**, Teufelsbauer H, Fritzer M, Kreuzer S, Knoflach P. Hemostasis activation in patients with liver cirrhosis. *Thromb Res* 1995; **77**: 271-278 [PMID: 7740519]

27   **Gulley D**, Teal E, Suvannasankha A, Chalasani N, Liangpunsakul S. Deep vein thrombosis and pulmonary embolism in cirrhosis patients. *Dig Dis Sci* 2008; **53**: 3012-3017 [PMID: 18443906 DOI: 10.1007/s10620-008-0265-3]

28   **Ambrosino P**, Tarantino L, Di Minno G, Paternoster M, Graziano V, Petitto M, Nasto A, Di Minno MN. The risk of venous thromboembolism in patients with cirrhosis. A systematic review and meta-analysis. *Thromb Haemost* 2017; **117**: 139-148 [PMID: 27761574 DOI: 10.1160/TH16-06-0450]

29   **Tsochatzis EA**, Senzolo M, Germani G, Gatt A, Burroughs AK. Systematic review: portal vein thrombosis in cirrhosis. *Aliment Pharmacol Ther* 2010; **31**: 366-374 [PMID: 19863496 DOI: 10.1111/j.1365-2036.2009.04182.x]

30   **Chawla YK**, Bodh V. Portal vein thrombosis. *J Clin Exp Hepatol* 2015; **5**: 22-40 [PMID: 25941431 DOI: 10.1016/j.jceh.2014.12.008]

31   **Fouzas I**, Paul A, Becker C, Vernadakis S, Treckmann JW, Máthé Z, Gerken G, Sotiropoulos GC. Orthotopic liver transplantation in patients with portal vein thrombosis in the absence of hepatocellular carcinoma. *Transplant Proc* 2012; **44**: 2734-2736 [PMID: 23146508 DOI: 10.1016/j.transproceed.2012.09.024]

32   **Senzolo M**, M Sartori T, Rossetto V, Burra P, Cillo U, Boccagni P, Gasparini D, Miotto D, Simioni P, Tsochatzis E, A Burroughs K. Prospective evaluation of anticoagulation and transjugular intrahepatic portosystemic shunt for the management of portal vein thrombosis in cirrhosis. *Liver Int* 2012; **32**: 919-927 [PMID: 22435854 DOI: 10.1111/j.1478-3231.2012.02785.x]

33   **Yamashita Y**, Bekki Y, Imai D, Ikegami T, Yoshizumi T, Ikeda T, Kawanaka H, Nishie A, Shirabe K, Maehara Y. Efficacy of postoperative anticoagulation therapy with enoxaparin for portal vein thrombosis after hepatic resection in patients with liver cancer. *Thromb Res* 2014; **134**: 826-831 [PMID: 25156238 DOI: 10.1016/j.thromres.2014.07.038]

34   **Duffy JP**, Hong JC, Farmer DG, Ghobrial RM, Yersiz H, Hiatt JR, Busuttil RW. Vascular complications of orthotopic liver transplantation: experience in more than 4,200 patients. *J Am Coll*

Surg 2009; **208**: 896-903; discussion 903-5 [PMID: 19476857 DOI: 10.1016/j.jamcollsurg.2008.12.032]

35   **Oh CK**, Pelletier SJ, Sawyer RG, Dacus AR, McCullough CS, Pruett TL, Sanfey HA. Uni- and multi-variate analysis of risk factors for early and late hepatic artery thrombosis after liver transplantation. *Transplantation* 2001; **71**: 767-772 [PMID: 11330540]

36   **Yang Y**, Zhao JC, Yan LN, Ma YK, Huang B, Yuan D, Li B, Wen TF, Wang WT, Xu MQ, Yang JY. Risk factors associated with early and late HAT after adult liver transplantation. *World J Gastroenterol* 2014; **20**: 10545-10552 [PMID: 25132774 DOI: 10.3748/wjg.v20.i30.10545]

37   **Silva MA**, Jambulingam PS, Gunson BK, Mayer D, Buckels JA, Mirza DF, Bramhall SR. Hepatic artery thrombosis following orthotopic liver transplantation: a 10-year experience from a single centre in the United Kingdom. *Liver Transpl* 2006; **12**: 146-151 [PMID: 16382467 DOI: 10.1002/lt.20566]

38   **Salami A**, Qureshi W, Kuriakose P, Moonka D, Yoshida A, Abouljoud M. Frequency and predictors of venous thromboembolism in orthotopic liver transplant recipients: a single-center retrospective review. *Transplant Proc* 2013; **45**: 315-319 [PMID: 23267811 DOI: 10.1016/j.transproceed.2012.06.060]

39   **Stine JG**, Pelletier SJ, Schmitt TM, Porte RJ, Northup PG. Pre-transplant portal vein thrombosis is an independent risk factor for graft loss due to hepatic artery thrombosis in liver transplant recipients. *HPB* (Oxford) 2016; **18**: 279-286 [PMID: 27017168 DOI: 10.1016/j.hpb.2015.10.008]

40   **Annamalai A**, Kim I, Sundaram V, Klein A. Incidence and risk factors of deep vein thrombosis after liver transplantation. *Transplant Proc* 2014; **46**: 3564-3569 [PMID: 25498090 DOI: 10.1016/j.transproceed.2014.09.113]

41   **Warnaar N**, Molenaar IQ, Colquhoun SD, Slooff MJ, Sherwani S, de Wolf AM, Porte RJ. Intraoperative pulmonary embolism and intracardiac thrombosis complicating liver transplantation: a systematic review. *J Thromb Haemost* 2008; **6**: 297-302 [PMID: 18005235 DOI: 10.1111/j.1538-7836.2007.02831.x]

42   **Sakai T**, Matsusaki T, Dai F, Tanaka KA, Donaldson JB, Hilmi IA, Wallis Marsh J, Planinsic RM, Humar A. Pulmonary thromboembolism during adult liver transplantation: incidence, clinical presentation, outcome, risk factors, and diagnostic predictors. *Br J Anaesth* 2012; **108**: 469-477 [PMID: 22174347 DOI: 10.1093/bja/aer392]

43   **Pereboom IT**, Adelmeijer J, van Leeuwen Y, Hendriks HG, Porte RJ, Lisman T. Development of a severe von Willebrand factor/ADAMTS13 dysbalance during orthotopic liver transplantation. *Am J Transplant* 2009; **9**: 1189-1196 [PMID: 19422343 DOI: 10.1111/j.1600-6143.2009.02621.x]

44   **Pereboom IT**, Lisman T, Porte RJ. Platelets in liver transplantation: friend or foe? *Liver Transpl* 2008; **14**: 923-931 [PMID: 18581510 DOI: 10.1002/lt.21510]

45   **Arshad F**, Lisman T, Porte RJ. Hypercoagulability as a contributor to thrombotic complications in the liver transplant recipient. *Liver Int* 2013; **33**: 820-827 [PMID: 23490221 DOI: 10.1111/liv.12140]

46   **Porte RJ**, Bontempo FA, Knot EA, Lewis JH, Kang YG, Starzl TE. Tissue-type-plasminogen-activator-associated fibrinolysis in orthotopic liver transplantation. *Transplant Proc* 1989; **21**: 3542 [PMID: 2500761]

47   **Lisman T**, Leebeek FW, Meijer K, Van Der Meer J, Nieuwenhuis HK, De Groot PG. Recombinant factor VIIa improves clot formation but not fibrolytic potential in patients with cirrhosis and during liver transplantation. *Hepatology* 2002; **35**: 616-621 [PMID: 11870375 DOI: 10.1053/jhep.2002.31771]

48   **Ganter MT**, Hofer CK. Coagulation monitoring: current techniques and clinical use of viscoelastic point-of-care coagulation devices. *Anesth Analg* 2008; **106**: 1366-1375 [PMID: 18420846 DOI: 10.1213/ane.0b013e318168b367]

49   **Lancé MD**. A general review of major global coagulation assays: thrombelastography, thrombin generation test and clot waveform analysis. *Thromb J* 2015; **13**: 1 [PMID: 25937820 DOI: 10.1186/1477-9560-13-1]

50   **Wikkelsø A**, Wetterslev J, Møller AM, Afshari A. Thromboelastography (TEG) or thromboelastometry (ROTEM) to monitor haemostatic treatment versus usual care in adults or children with bleeding. *Cochrane Database Syst Rev* 2016; : CD007871 [PMID: 27552162 DOI: 10.1002/14651858.CD007871. pub3]

51   **Forkin KT**, Colquhoun DA, Nemergut EC, Huffmyer JL. The Coagulation Profile of End-Stage Liver Disease and Considerations for Intraoperative Management. *Anesth Analg* 2018; **126**: 46-61 [PMID: 28795966 DOI: 10.1213/ANE.0000000000002394]

52   **Hincker A**, Feit J, Sladen RN, Wagener G. Rotational thromboelastometry predicts thromboembolic complications after major non-cardiac surgery. *Crit Care* 2014; **18**: 549 [PMID: 25292221 DOI: 10.1186/s13054-014-0549-2]

53   **Kashuk JL**, Moore EE, Sabel A, Barnett C, Haenel J, Le T, Pezold M, Lawrence J, Biffl WL, Cothren CC, Johnson JL. Rapid thrombelastography (r-TEG) identifies hypercoagulability and predicts thromboembolic events in surgical patients. *Surgery* 2009; **146**: 764-72; discussion 772-4 [PMID: 19789037 DOI: 10.1016/j.surg.2009.06.054]

54   **Cerutti E**, Stratta C, Romagnoli R, Schellino MM, Skurzak S, Rizzetto M, Tamponi G, Salizzoni M. Thromboelastogram monitoring in the perioperative period of hepatectomy for adult living liver donation. *Liver Transpl* 2004; **10**: 289-294 [PMID: 14762869 DOI: 10.1002/lt.20078]

55   **Krzanicki D**, Sugavanam A, Mallett S. Intraoperative hypercoagulability during liver transplantation as demonstrated by thromboelastography. *Liver Transpl* 2013; **19**: 852-861 [PMID: 23696318 DOI: 10.1002/lt.23668]

56   **McCrath DJ**, Cerboni E, Frumento RJ, Hirsh AL, Bennett-Guerrero E. Thromboelastography maximum amplitude predicts postoperative thrombotic complications including myocardial infarction. *Anesth Analg* 2005; **100**: 1576-1583 [PMID: 15920177 DOI: 10.1213/01.ANE.0000155290.86795.12]

57   **Rafiq S**, Johansson PI, Ostrowski SR, Stissing T, Steinbrüchel DA. Hypercoagulability in patients undergoing coronary artery bypass grafting: prevalence, patient characteristics and postoperative outcome. *Eur J Cardiothorac Surg* 2012; **41**: 550-555 [PMID: 22011771 DOI: 10.1093/ejcts/ezr001]

58   **Lerner AB**, Sundar E, Mahmood F, Sarge T, Hanto DW, Panzica PJ. Four cases of cardiopulmonary thromboembolism during liver transplantation without the use of antifibrinolytic drugs. *Anesth Analg* 2005; **101**: 1608-1612 [PMID: 16301227 DOI: 10.1213/01.ANE.0000184256.28981.2B]

59   **Zanetto A**, Senzolo M, Vitale A, Cillo U, Radu C, Sartorello F, Spiezia L, Campello E, Rodriguez-Castro K, Ferrarese A, Farinati F, Burra P, Simioni P. Thromboelastometry hypercoagulable profiles and portal vein thrombosis in cirrhotic patients with hepatocellular carcinoma. *Dig Liver Dis* 2017; **49**: 440-445 [PMID: 28109767 DOI: 10.1016/j.dld.2016.12.019]

60   **Zahr Eldeen F**, Roll GR, Derosas C, Rao R, Khan MS, Gunson BK, Hodson J, Mergental H, Ferraz-Neto BH, Isaac J, Muiesan P, Mirza DF, Iqbal A, Perera MT. Preoperative Thromboelastography as a Sensitive Tool Predicting Those at Risk of Developing Early Hepatic Artery Thrombosis After Adult Liver Transplantation. *Transplantation* 2016; **100**: 2382-2390 [PMID: 27780186 DOI: 10.1097/TP.0000000000001395]

61   **Blasi A**, Molina V, Sanchez-Cabús S, Balust J, Garcia-Valdecasas JC, Taura P. Prediction of thromboembolic complications after liver resection for cholangiocarcinoma: is there a place for thromboelastometry? *Blood Coagul Fibrinolysis* 2018; **29**: 61-66 [PMID: 29045240 DOI: 10.1097/MBC.0000000000000672]

62   **Lentschener C**, Flaujac C, Ibrahim F, Gouin-Thibault I, Bazin M, Sogni P, Samama CM. Assessment of haemostasis in patients with cirrhosis: Relevance of the ROTEM tests?: A prospective, cross-sectional study. *Eur J Anaesthesiol* 2016; **33**: 126-133 [PMID: 26258657 DOI: 10.1097/EJA.0000000000000322]

63   **De Pietri L**, Bianchini M, Rompianesi G, Bertellini E, Begliomini B. Thromboelastographic reference ranges for a cirrhotic patient population undergoing liver transplantation. *World J Transplant*

2016; **6**: 583-593 [PMID: 27683637 DOI: 10.5500/wjt.v6.i3.583]

64   **Lang T**, von Depka M. [Possibilities and limitations of thrombelastometry/-graphy]. *Hamostaseologie* 2006; **26**: S20-S29 [PMID: 16953288]

65   **Jámbor C**, Reul V, Schnider TW, Degiacomi P, Metzner H, Korte WC. In vitro inhibition of factor XIII retards clot formation, reduces clot firmness, and increases fibrinolytic effects in whole blood. *Anesth Analg* 2009; **109**: 1023-1028 [PMID: 19762725 DOI: 10.1213/ANE.0b013e3181b5a263]

66   **Kitchen DP**, Kitchen S, Jennings I, Woods TA, Fitzmaurice DA, Murray ET, Walker ID. Point of Care INR testing devices: performance of the Roche CoaguChek XS and XS Plus in the UK NEQAS BC external quality assessment programme for healthcare professionals: four years' experience. *J Clin Pathol* 2012; **65**: 1119-1123 [PMID: 23038688 DOI: 10.1136/jclinpath-2012-201049]

67   **MacDonald SG**, Luddington RJ. Critical factors contributing to the thromboelastography trace. *Semin Thromb Hemost* 2010; **36**: 712-722 [PMID: 20978992 DOI: 10.1055/s-0030-1265288]

68   **Tripodi A**. Thrombin Generation Assay and Its Application in the Clinical Laboratory. *Clin Chem* 2016; **62**: 699-707 [PMID: 26955824 DOI: 10.1373/clinchem.2015.248625]

69   **Hemker HC**, Al Dieri R, De Smedt E, Béguin S. Thrombin generation, a function test of the haemostatic-thrombotic system. *Thromb Haemost* 2006; **96**: 553-561 [PMID: 17080210]

70   **Dielis AW**, Castoldi E, Spronk HM, van Oerle R, Hamulyák K, Ten Cate H, Rosing J. Coagulation factors and the protein C system as determinants of thrombin generation in a normal population. *J Thromb Haemost* 2008; **6**: 125-131 [PMID: 17988231 DOI: 10.1111/j.1538-7836.2007.02824.x]

71   **Kaneko J**, Sugawara Y, Tamura S, Togashi J, Matsui Y, Akamatsu N, Kishi Y, Makuuchi M. Coagulation and fibrinolytic profiles and appropriate use of heparin after living-donor liver transplantation. *Clin Transplant* 2005; **19**: 804-809 [PMID: 16313329 DOI: 10.1111/j.1399-0012.2005.00425.x]

72   **Stange BJ**, Glanemann M, Nuessler NC, Settmacher U, Steinmüller T, Neuhaus P. Hepatic artery thrombosis after adult liver transplantation. *Liver Transpl* 2003; **9**: 612-620 [PMID: 12783404 DOI: 10.1053/jlts.2003.50098]

73   **Yip J**, Bruno DA, Burmeister C, Kazimi M, Yoshida A, Abouljoud MS, Schnickel GT. Deep Vein Thrombosis and Pulmonary Embolism in Liver Transplant Patients: Risks and Prevention. *Transplant Direct* 2016; **2**: e68 [PMID: 27500259 DOI: 10.1097/TXD.0000000000000578]

74   **Gad EH**, Abdelsamee MA, Kamel Y. Hepatic arterial and portal venous complications after adult and pediatric living donor liver transplantation, risk factors, management and outcome (A retrospective cohort study). *Ann Med Surg* (Lond) 2016; **8**: 28-39 [PMID: 27257483 DOI: 10.1016/j.amsu.2016.04.021]

75   **Uchikawa Y**, Ikegami T, Masuda Y, Ohno Y, Mita A, Urata K, Nakazawa Y, Terada M, Miyagawa S. Administration of dalteparin based on the activated clotting time for prophylaxis of hepatic vessel thrombosis in living donor liver transplantation. *Transplant Proc* 2009; **41**: 3784-3790 [PMID: 19917388 DOI: 10.1016/j.transproceed.2009.04.011]

76   **Sugawara Y**, Kaneko J, Akamatsu N, Imamura H, Kokudo N, Makuuchi M. Anticoagulant therapy against hepatic artery thrombosis in living donor liver transplantation. *Transplant Proc* 2002; **34**: 3325-3326 [PMID: 12493462]

77   **Wolf DC**, Freni MA, Boccagni P, Mor E, Chodoff L, Birnbaum A, Miller CM, Schwartz ME, Bodenheimer HC Jr. Low-dose aspirin therapy is associated with few side effects but does not prevent hepatic artery thrombosis in liver transplant recipients. *Liver Transpl Surg* 1997; **3**: 598-603 [PMID: 9404960]

78   **Vivarelli M**, La Barba G, Cucchetti A, Lauro A, Del Gaudio M, Ravaioli M, Grazi GL, Pinna AD. Can antiplatelet prophylaxis reduce the incidence of hepatic artery thrombosis after liver transplantation? *Liver Transpl* 2007; **13**: 651-654 [PMID: 17457885 DOI: 10.1002/lt.21028]

79   **Shay R**, Taber D, Pilch N, Meadows H, Tischer S, McGillicuddy J, Bratton C, Baliga P, Chavin K. Early aspirin therapy may reduce

hepatic artery thrombosis in liver transplantation. *Transplant Proc* 2013; **45**: 330-334 [PMID: 23267805 DOI: 10.1016/j.transproceed. 2012.05.075]

80 **Mori A**, Iida T, Iwasaki J, Ogawa K, Fujimoto Y, Uemura T, Hatano E, Okajima H, Kaido T, Uemoto S. Portal vein reconstruction in adult living donor liver transplantation for patients with portal vein thrombosis in single center experience. *J Hepatobiliary Pancreat Sci* 2015; **22**: 467-474 [PMID: 25755116 DOI: 10.1002/jhbp.235]

81 **Hugenholtz GC**, Northup PG, Porte RJ, Lisman T. Is there a rationale for treatment of chronic liver disease with antithrombotic therapy? *Blood Rev* 2015; **29**: 127-136 [PMID: 25468718 DOI: 10.1016/j.blre.2014.10.002]

82 **Stahl RL**, Duncan A, Hooks MA, Henderson JM, Millikan WJ, Warren WD. A hypercoagulable state follows orthotopic liver transplantation. *Hepatology* 1990; **12**: 553-558 [PMID: 2401460]

83 **Lisman T**, Bakhtiari K, Pereboom IT, Hendriks HG, Meijers JC, Porte RJ. Normal to increased thrombin generation in patients undergoing liver transplantation despite prolonged conventional coagulation tests. *J Hepatol* 2010; **52**: 355-361 [PMID: 20132999 DOI: 10.1016/j.jhep.2009.12.001]

84 **Mallett SV**, Sugavanam A, Krzanicki DA, Patel S, Broomhead RH, Davidson BR, Riddell A, Gatt A, Chowdary P. Alterations in coagulation following major liver resection. *Anaesthesia* 2016; **71**: 657-668 [PMID: 27030945 DOI: 10.1111/anae.13459]

85 **Potze W**, Alkozai EM, Adelmeijer J, Porte RJ, Lisman T. Hypercoagulability following major partial liver resection - detected by thrombomodulin-modified thrombin generation testing. *Aliment Pharmacol Ther* 2015; **41**: 189-198 [PMID: 25382796 DOI: 10.1111/apt.13022]

86 **Remuzzi G**, Bertani T. Renal vascular and thrombotic effects of cyclosporine. *Am J Kidney Dis* 1989; **13**: 261-272 [PMID: 2650537]

87 **Gómez Cuervo C**, Bisbal Pardo O, Pérez-Jacoiste Asín MA. Efficacy and safety of the use of heparin as thromboprophylaxis in patients with liver cirrhosis: a systematic review and meta-analysis. *Thromb Res* 2013; **132**: 414-419 [PMID: 23993900 DOI: 10.1016/j.thromres.2013.08.001]

88 **Intagliata NM**, Henry ZH, Shah N, Lisman T, Caldwell SH, Northup PG. Prophylactic anticoagulation for venous thromboembolism in hospitalized cirrhosis patients is not associated with high rates of gastrointestinal bleeding. *Liver Int* 2014; **34**: 26-32 [PMID: 23758818 DOI: 10.1111/liv.12211]

89 **Intagliata NM**, Northup PG. Anticoagulant Therapy in Patients with Cirrhosis. *Semin Thromb Hemost* 2015; **41**: 514-519 [PMID: 26049069 DOI: 10.1055/s-0035-1550436]

90 **Intagliata NM**, Maitland H, Caldwell SH. Direct Oral Anticoagulants in Cirrhosis. *Curr Treat Options Gastroenterol* 2016; **14**: 247-256 [PMID: 27020266 DOI: 10.1007/s11938-016-0092-0]

91 **Massicotte L**, Denault AY, Beaulieu D, Thibeault L, Hevesi Z, Nozza A, Lapointe R, Roy A. Transfusion rate for 500 consecutive liver transplantations: experience of one liver transplantation center. *Transplantation* 2012; **93**: 1276-1281 [PMID: 22617090 DOI: 10.1097/TP.0b013e318250fc25]

92 **Agnelli G**, Borm J, Cosmi B, Levi M, ten Cate JW. Effects of standard heparin and a low molecular weight heparin (Kabi 2165) on fibrinolysis. *Thromb Haemost* 1988; **60**: 311-313 [PMID: 2851195]

93 **Padilla A**, Gray E, Pepper DS, Barrowcliffe TW. Inhibition of thrombin generation by heparin and low molecular weight (LMW) heparins in the absence and presence of platelet factor 4 (PF4). *Br J Haematol* 1992; **82**: 406-413 [PMID: 1329921]

94 **Ha NB**, Regal RE. Anticoagulation in Patients With Cirrhosis: Caught Between a Rock-Liver and a Hard Place. *Ann Pharmacother* 2016; **50**: 402-409 [PMID: 26861989 DOI: 10.1177/1060028016631760]

95 **Fuentes A**, Gordon-Burroughs S, Hall JB, Putney DR, Monsour HP Jr. Comparison of anti-Xa and activated partial thromboplastin time monitoring for heparin dosing in patients with cirrhosis. *Ther Drug Monit* 2015; **37**: 40-44 [PMID: 24901494 DOI: 10.1097/FTD.0000000000000105]

96 **Potze W**, Arshad F, Adelmeijer J, Blokzijl H, van den Berg AP, Porte RJ, Lisman T. Routine coagulation assays underestimate levels of antithrombin-dependent drugs but not of direct anticoagulant drugs in plasma from patients with cirrhosis. *Br J Haematol* 2013; **163**: 666-673 [PMID: 24219333 DOI: 10.1111/bjh.12593]

97 **Assfalg V**, Hüser N. Heparin-induced thrombocytopenia in solid organ transplant recipients: The current scientific knowledge. *World J Transplant* 2016; **6**: 165-173 [PMID: 27011914 DOI: 10.5500/wjt.v6.i1.165]

98 **Zaman S**, Wiebe S, Bernal W, Wendon J, Czuprynska J, Auzinger G. Increased prevalence of heparin-induced thrombocytopenia in patients with Budd-Chiari syndrome: a retrospective analysis. *Eur J Gastroenterol Hepatol* 2016; **28**: 967-971 [PMID: 27015137 DOI: 10.1097/MEG.0000000000000632]

99 **Weitz JI**. Low-molecular-weight heparins. *N Engl J Med* 1997; **337**: 688-698 [PMID: 9278467 DOI: 10.1056/NEJM199709043371007]

100 **Senzolo M**, Rodriguez-Castro KI, Rossetto V, Radu C, Gavasso S, Carraro P, Zerbinati P, Sartori MT, Simioni P. Increased anticoagulant response to low-molecular-weight heparin in plasma from patients with advanced cirrhosis. *J Thromb Haemost* 2012; **10**: 1823-1829 [PMID: 22712870 DOI: 10.1111/j.1538-7836.2012.04824.x]

101 **Bounameaux H**, de Moerloose P. Is laboratory monitoring of low-molecular-weight heparin therapy necessary? No. *J Thromb Haemost* 2004; **2**: 551-554 [PMID: 15102007 DOI: 10.1111/j.1538-7933.2004.00648.x]

102 **Bechmann LP**, Sichau M, Wichert M, Gerken G, Kröger K, Hilgard P. Low-molecular-weight heparin in patients with advanced cirrhosis. *Liver Int* 2011; **31**: 75-82 [PMID: 20958919 DOI: 10.1111/j.1478-3231.2010.02358.x]

103 **Linkins LA**, Julian JA, Rischke J, Hirsh J, Weitz JI. In vitro comparison of the effect of heparin, enoxaparin and fondaparinux on tests of coagulation. *Thromb Res* 2002; **107**: 241-244 [PMID: 12479885]

104 **Henry TD**, Satran D, Knox LL, Iacarella CL, Laxson DD, Antman EM. Are activated clotting times helpful in the management of anticoagulation with subcutaneous low-molecular-weight heparin? *Am Heart J* 2001; **142**: 590-593 [PMID: 11579347 DOI: 10.1067/mhj.2001.117317]

105 **Marmur JD**, Anand SX, Bagga RS, Fareed J, Pan CM, Sharma SK, Richard MF. The activated clotting time can be used to monitor the low molecular weight heparin dalteparin after intravenous administration. *J Am Coll Cardiol* 2003; **41**: 394-402 [PMID: 12575965]

106 **Potze W**, Arshad F, Adelmeijer J, Blokzijl H, van den Berg AP, Meijers JC, Porte RJ, Lisman T. Differential in vitro inhibition of thrombin generation by anticoagulant drugs in plasma from patients with cirrhosis. *PLoS One* 2014; **9**: e88390 [PMID: 24505487 DOI: 10.1371/journal.pone.0088390]

107 **Fritsma GA**, Ens GE, Alvord MA, Carroll AA, Jensen R. Monitoring the antiplatelet action of aspirin. *JAAPA* 2001; **14**: 57-58, 61-62 [PMID: 11523339]

108 **Hugenholtz GG**, Porte RJ, Lisman T. The platelet and platelet function testing in liver disease. *Clin Liver Dis* 2009; **13**: 11-20 [PMID: 19150305 DOI: 10.1016/j.cld.2008.09.010]

109 **De Lédinghen V**, Heresbach D, Fourdan O, Bernard P, Liebaert-Bories MP, Nousbaum JB, Gourlaouen A, Becker MC, Ribard D, Ingrand P, Silvain C, Beauchant M. Anti-inflammatory drugs and variceal bleeding: a case-control study. *Gut* 1999; **44**: 270-273 [PMID: 9895389]

110 **European Association for the Study of the Liver.**. EASL clinical practice guidelines on the management of ascites, spontaneous bacterial peritonitis, and hepatorenal syndrome in cirrhosis. *J Hepatol* 2010; **53**: 397-417 [PMID: 20633946 DOI: 10.1016/j.jhep.2010.05.004]

111 **Burger W**, Chemnitius JM, Kneissl GD, Rücker G. Low-dose aspirin for secondary cardiovascular prevention - cardiovascular risks after its perioperative withdrawal versus bleeding risks with its continuation - review and meta-analysis. *J Intern Med* 2005; **257**: 399-414 [PMID: 15836656 DOI: 10.1111/j.1365-2796.2005.01477.x]

112 **Chernoguz A**, Telem DA, Chu E, Ozao-Choy J, Tammaro Y, Divino CM. Cessation of clopidogrel before major abdominal procedures. *Arch Surg* 2011; **146**: 334-339 [PMID: 21422366 DOI: 10.1001/archsurg.2011.23]

113 **Hum J**, Shatzel JJ, Jou JH, Deloughery TG. The efficacy and safety of direct oral anticoagulants vs traditional anticoagulants in cirrhosis. *Eur J Haematol* 2017; **98**: 393-397 [PMID: 28009449 DOI: 10.1111/ejh.12844]

MicroRNAs in the prognosis and therapy of colorectal cancer: From bench to bedside

Kenneth KW To, Christy WS Tong, Mingxia Wu, William CS Cho

Kenneth KW To, Christy WS Tong, Mingxia Wu, School of Pharmacy, Faculty of Medicine, the Chinese University of Hong Kong, Hong Kong, China

William CS Cho, Department of Clinical Oncology, Queen Elizabeth Hospital, Hong Kong, China

ORCID number: Kenneth KW To (0000-0003-2755-0283); Christy WS Tong (0000-0003-2010-1418); Mingxia Wu (0000-0002-6070-5569); William CS Cho (0000-0003-4174-4586).

Abstract

MicroRNAs (miRNAs) are small, single-stranded, noncoding RNAs that can post-transcriptionally regulate the expression of various oncogenes and tumor suppressor genes. Dysregulated expression of many miRNAs have been shown to mediate the signaling pathways critical in the multistep carcinogenesis of colorectal cancer (CRC). MiRNAs are stable and protected from RNase-mediated degradation, thereby enabling its detection in biological fluids and archival tissues for biomarker studies. This review focuses on the role and application of miRNAs in the prognosis and therapy of CRC. While stage II CRC is potentially curable by surgical resection, a significant percentage of stage II CRC patients do develop recurrence. MiRNA biomarkers may be used to stratify such high-risk population for adjuvant chemotherapy to provide better prognoses. Growing evidence also suggests that miRNAs are involved in the metastatic process of CRC. Certain of these miRNAs may thus be used as prognostic biomarkers to identify patients more likely to have micro-metastasis, who could be monitored more closely after surgery and/or given more aggressive adjuvant chemotherapy. Intrinsic and acquired resistance to chemotherapy severely hinders successful chemotherapy in CRC treatment. Predictive miRNA biomarkers for response to chemotherapy may identify patients who will benefit the most from a particular regimen and also spare the patients from unnecessary side effects. Selection of patients to receive the new targeted therapy is becoming possible with the use of predictive miRNA biomarkers. Lastly, forced expression of tumor suppressor miRNA or silencing of oncogenic miRNA in tumors by gene therapy can also be adopted to treat CRC alone or in combination with other

chemotherapeutic drugs.

Key words: MicroRNA; Colorectal cancer; Multidrug resistance; Prognosis; Therapeutic target; Apoptosis; Metastasis; Recurrence; Risk stratification

Core tip: MicroRNAs (miRNAs) are important mediators regulating the initiation, progression, metastasis and recurrence of colorectal cancer (CRC). Numerous studies have reported dysregulation of miRNAs in tumor specimens and body fluids, including serum, plasma and feces. Furthermore, the miRNAs were more recently found in circulating exosomes from CRC patients. Fortunately, this finding suggests potential diagnostic, prognostic and therapeutic applications of miRNAs at different stages of CRC. In this review, we aim to outline the current body of knowledge pertaining to the critical roles played by miRNAs in the molecular pathogenesis of CRC. Our focus is to delineate practical applications of miRNAs as prognostic biomarkers and therapeutic targets in the treatment of CRC.

INTRODUCTION

Colorectal cancer (CRC) is the 3rd most common cancer and the 3rd leading cause of cancer-related deaths worldwide[1]. For 2018, there is an estimated incidence of over 140000 new cases and mortality of over 50000 annually in the United States[2]. It is widely believed that CRC develops in multi-step process, from aberrant crypt foci, through benign precancerous lesions (adenomas), to malignant tumors (adenocarcinomas) over an extended period of time[3]. The majority of CRC is sporadic, though approximately 20%-30% of CRC patients carry inherited mutations[4,5]. Accumulation of numerous genetic mutations and/or epigenetic changes is required to drive the carcinogenic progression through progressive functional disruption of tumor suppressor genes and oncogenes. While the prognosis for advanced CRC remains dismal, the disease is curable in its early stages, thereby highlighting the importance of prevention and early detection.

Treatment of CRC usually involves surgical resection of the primary tumor(s) followed by chemotherapy and/or targeted therapy for the advanced (stage III and IV) disease[6]. New drugs approved for CRC in recent years mainly fall into the class of molecular targeted drugs, including the vascular endothelial growth factor receptor (VEGFR) and epidermal growth factor receptor (EGFR) inhibitors[7].

MicroRNAs (miRNAs) are short (~22 nucleotides in length) endogenous non-coding RNAs that repress gene expression in eukaryotic organisms. There are numerous studies reporting the correlation between dysregulated miRNAs and the aberrant regulation of signaling pathways involved in CRC initiation and progression. Michael *et al*[8] was the first to report a dramatic downregulation of miR-143 and miR-145 in CRC relative to normal colon epithelial cells, thereby suggesting a role of miRNAs in CRC pathogenesis. Other studies have demonstrated the tumor suppressive or oncogenic functions of miRNAs in CRC. Therefore, miRNAs might have diagnostic and prognostic value for CRC patients. MiRNAs may also represent novel therapeutic targets for gene therapy in the treatment of CRC.

In this review, the dysregulation of miRNAs leading to the initiation, progression and metastasis of CRC will be discussed. We also describe the translation of miRNA research to potential prognostic and therapeutic applications in the management of CRC in clinical settings.

MIRNAS AND DYSREGULATION OF SIGNALING PATHWAYS IN CRC

Gene regulation by miRNAs is mediated by the formation of hybrids with the 3-untranslated region (3'UTR) sequences of the target mRNAs, leading to mRNA degradation and/or translational inhibition[9]. MiRNAs play key roles in numerous cellular processes, including cell proliferation, differentiation, apoptosis, and development. These miRNAs are observed to regulate the expression of approximately one-third of human protein-coding genes[10].

miRNAs and WNT/β-catenin pathway

The dysregulation of the Wnt/β-catenin pathway is one of the earliest events driving CRC carcinogenesis. Constitutively active β-catenin upregulates the expression of Wnt target genes transcriptionally to initiate CRC formation[11]. There is mounting evidence suggesting there is crosstalk between miRNAs and the Wnt/β-catenin pathway in CRC development. While miRNAs were found to activate or inhibit the canonical Wnt pathway, Wnt activation was also shown to increase expression of miRNAs by directly binding to their gene promoters.

MiR-224 was recently shown to activate the Wnt/β-catenin signaling and direct the nuclear translocation of β-catenin in CRC by downregulating GSK3β and SFRP2[12]. Knockdown of miR-224 was found to restore the expression of GSK3β and SFRP2 and inhibit Wnt/β-catenin-mediated cell metastasis and cell proliferation.

Cancer stem cells (CSCs) are widely believed to be the key driving force for the initiation of cancer. CSC

subpopulations, capable of self-renewal and having the ability to initiate and sustain tumor growth, metastasis and resistance to chemotherapy, have been identified in CRC[13]. The Wnt pathway plays a key role in the induction of symmetrical cell division of CSCs, which disrupts the homeostasis of normal stem cells and leads to cancer formation[14,15]. To this end, miR-146a was shown to activate the Wnt pathway in colorectal cancer CSCs to stabilize β-catenin, thereby regulating the symmetrical cell division to promote CRC initiation and progression[16]. Interestingly, miR-146a was itself activated transcriptionally by Snail *via* a β-catenin-TCF4 complex[16]. Therefore, a feedback loop consisting of Snail-miR-146a-β-catenin is operating in CSC of colorectal cancer to maintain Wnt activity.

miRNAs and EGFR pathways
In recent years, anti-EGFR targeted drugs have been approved for treating metastatic CRC. However, treatment response is hindered by dysregulation of the PI3K/AKT and KRAS/RAF/ERK pathways downstream of EGFR.

In the PI3K/AKT pathway, mutation of the *PIK3CA* gene has been demonstrated in 15%-20% of CRC cases[17]. Interestingly, mutations in the miR-520a and miR-525a binding sites at the 3'UTR of PIK3CA were found to enhance the sensitivity of CRC cell lines to saracatinib, which inhibits the activation of Akt-dependent signaling[18]. Conversely, reduced expression of miR-126 has been shown to mediate amplification of the PI3K/AKT signal in CRC[19].

Activating KRAS mutations constitutes up to 30%-60% of all CRC cases[20], which mediates primary resistance to anti-EGFR targeted therapy. Moreover, even in CRC bearing wild-type KRAS, the response to anti-EGFR targeted therapy is less than 40%[21]. Additional molecular signature(s) are needed for the selection of patients who respond well to anti-EGFR targeted therapy. To this end, miRNAs such as let-7[22], miR-18a*[23], miR-30b[24], miR-143[25], and miR-145[26] have been identified as tumor suppressors that inhibit KRAS expression. These miRNAs may be used as biomarkers to predict favorable response in patients to anti-EGFR therapy.

miRNAs and TGF-β signaling pathway
Transforming growth factor-beta (TGF-β) plays a key role in inhibiting cell proliferation, and it also modulates tumor invasion and tumor microenvironment modification. It has been estimated that 30% of CRC cases are due to mutations in TGF-β type II receptor (TGFβR2)[27,28]. TGF-β binds to its receptors (TGF-βR) and mediates the activation of its downstream pathway through phosphorylation of Smad. The complex is subsequently translocated to the nucleus, and it regulates the expression of transcriptional factors, including Snail, ZEB and Twist. Several miRNAs, including miR-21[29], miR-106a[30], and miR-301a[31], have been reported to induce

stemness or promote cancer migration and invasion in CRC by targeting the TGF-β/Smad signaling pathway. Conversely, decreased expression of miR-25 in CRC cell lines was also found to activate SMAD7, which is a negative regulator of TGF-β signaling pathway, to promote cancer proliferation and metastasis[32]. Interestingly, miR-187, a validated downstream effector of the TGFβ pathway, was shown to suppress Smad-mediated epithelial-mesenchymal transition (EMT) in CRC cells[33].

miRNAs and epithelial-to-mesenchymal transition (EMT)
The activation of EMT, a cellular process of converting polarized epithelial cells to mesenchymal cells, enables cancer cells to migrate and invade into metastatic sites[34]. A number of miRNAs have been identified as key regulators of this EMT process in CRC[35]. MiR-29c has been shown to be remarkably downregulated in primary CRC with distant metastasis and it was associated with significantly shorter patient survival[36]. Importantly, forced expression of miR-29c was demonstrated to inhibit cell migration and invasion *in vitro* and metastasis *in vivo*[36], which is related to its inhibition of the ERK/GSK3β/β-catenin and AKT/GSK3β/β-catenin pathways. Conversely, liver metastatic tissues were found to express higher level of miR-200c than the primary CRC tumor[37]. This increased expression has been specifically associated with hypomethylation of the promoter of the miRNA gene. The Wnt/β-catenin pathway was also shown to transactivate miR-150, which subsequently promoted EMT of CRC cells by suppressing CREB signaling[38].

MIRNAS AS POTENTIAL CLINICAL BIOMARKERS IN CRC

miRNAS as diagnostic biomarkers for CRC
It is widely believed that a series of sequential genetic changes are needed to drive the conversion of normal colonic epithelium to malignant CRC. If the precancerous adenoma can be detected and treated prior to the development of advanced carcinoma, then patient mortality would be reduced[39]. While colonoscopy remains the gold standard screening test for the diagnosis of CRC[39,40], the application of miRNA as diagnostic markers for CRC has attracted a lot of attention in recent years. Two of the first few miRNAs identified in CRC tumor specimens, miR-143 and miR-145, were, relative to normal colonic mucosa, found to be consistently downregulated in adenoma and other stages of CRC[8,41]. More recently, several other miRNAs (miR-21, miR-29a, miR-92a and miR-135b) have also been shown to, when compared to normal tissues, be upregulated in patients with high-risk adenomas[42]. A few excellent review articles about the development of miRNA-based biomarkers for the diagnosis of CRC can be found in recent literature[43-47]. Our review will focus more on the prognostic application and prediction of

therapeutic response by miRNAs in CRC.

miRNA as prognostic biomarkers for CRC

Although tissue-specific signatures of miRNAs have been reported[48,49], more work is still needed to define a set of differentially expressed miRNAs suitable for screening CRC in the clinical setting. Besides using miRNAs as a tool to screen for early adenomas and CRC cases, it may be more useful to use miRNAs as a prognostic tool to guide treatment for CRC patients.

miRNAs in tumor tissues

Since surgical resection of the primary tumor is the treatment of choice in early stages of CRC, tumor tissue can be available as an important source for the identification of CRC-related miRNAs. Numerous miRNAs have been reported to be upregulated or downregulated in CRC cell lines in tumor specimens from patients, suggesting their association with patients' prognosis and response to anticancer drugs. Table 1 summarizes representative miRNAs of prognostic value in CRC tissues.

It is known that oncogenic miRNAs (also known as oncomiRs) and tumor suppressor miRNAs are differentially expressed during the development and progression of CRC. Moreover, miRNA expression in CRC is regulated in stage-specific manner (Figure 1). Therefore, tracing the order of miRNA regulation during CRC progression may indicate disease prognosis and predict treatment response. To this end, let-7, miR-21, miR-29a and miR-17-92 cluster were of particular importance.

Let-7 was known to regulate the expression of RAS and MYC genes, both of which are key factors mediating CRC progression and metastasis. Interestingly, the let-7 miRNA single nucleotide polymorphism (SNP) in the K-Ras 3'UTR has been reported to be prognostic in early-stage CRC (see below for more discussion about miRNA binding site SNP)[50]. MiR-17-92 is a highly conserved cluster that gives rise to six mature miRNAs, including miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92-1. This cluster is frequently amplified in CRC, suggesting an oncogenic role for these miRNAs[51]. Moreover, miR-17 and Tumor, Node, and Metastasis (TNM) staging were shown to be significant but independent prognostic biomarkers in CRC patients[51]. MiR-92a, targeting the anti-apoptotic protein BIM, was found to be expressed at higher level in both adenomas and carcinoma[52]. The other miRNAs within this cluster were shown to regulate multiple targets to drive CRC progression (PTEN – miR-19a and miR-19b-1; BCL2L11 – miR-19a, miR-19b-1, miR-20a, miR-92; E2F1 – miR-17 and miR-20a; TGF-β receptor II – miR-17 and miR-20a[53-56]).

The remarkable upregulation of miR-21 in CRC has advocated its function as a prognostic marker[57]. In a small cohort of 29 CRC patients, high level of miR-21 in the tumor specimens was found to be associated with lymph node metastasis and also distal metastasis[58].

In a bigger cohort of 156 CRC cases, elevated level of miR-21 was also found to be correlated with venous invasion, liver metastasis and advanced Dukes' stage[59]. A differentially expressed microRNA signature was recently reported in American CRC patients with liver metastasis[60]. miR-21, together with miR-93 and miR-103, were found to be increased in advanced CRC with liver metastasis.

The risk of CRC recurrence was found to be associated with high expression of miR-29a in tumor tissues in patients with stage II CRC[61]. miR-29a was later identified as a novel metastasis-promoting factor through upregulation of matrix metalloproteinase 2 and downregulation of E-cadherin *via* targeting the tumor suppressor gene KLF4[62].

LIMITATION WITH MEASURING MIRNAS IN ARCHIVAL TUMOR TISSUES

The major limitation of detecting miRNAs in archival tumor tissues is heterogeneity (from within the same primary tumor and also between different metastatic sites). Therefore, evaluating miRNA expression from serum/plasma (*i.e.* circulating miRNAs) may be potentially preferable for predicting prognosis in the clinical setting.

Circulating miRNAs

For CRC, the traditionally used blood-based biomarkers, including carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9), suffer from low sensitivity, particularly for early stage CRC[63]. In recent years, miRNA-based biomarkers have shown promise as CRC-specific prognostic tests due to their high sensitivity and specificity. MiRNAs are remarkably stable in blood despite the high level of ribonuclease that rapidly degrade RNA[64]. MiRNAs are protected from enzyme degradation because they are stored in exosomes or vesicles, or bound to lipoproteins[65,66].

Several miRNAs isolated from serum have been evaluated for their use as prognostic markers in CRC (Table 2). A recent report revealed a panel of miRNAs (miR-21, let-7g, miR-31, miR-92a, miR-181b, and miR-203) that can be reliably used as prognostic marker for CRC, with 93% sensitivity and 91% specificity in comparison with the traditional markers (CEA and CA19-9)[67]. Furthermore, miR-21 has also been shown to be able to differentiate both adenoma and CRC from healthy controls[68,69]. The detection of miR-141 in plasma samples has been used as a supplementary prognostic marker besides CEA for the detection of CRC patients with distant metastasis[70]. Moreover, the changes in plasma levels of miR-24, miR-320a and miR423-5p can be evaluated to predict the risk of post-surgery metastasis in CRC patients[71].

Fecal-based miRNAs

MiRNAs are known to be sufficiently stable for

Table 1 A list of representative miRNAs identified in tumor tissues that are of prognostic value in colorectal cancer patients

miRNA	Method of detection	Patient number	Observations and correlation with clinical outcome	Ref.
miR-15a/miR-16	qRT-PCR	126	miR-15a/miR-16 downregulation were significantly associated with advanced TNM staging, poorly histological grade, positive lymph node metastasis. miR-15a/miR-16 combination were identified as independent predictors of unfavorable OS and DFS	[183]
miR-17-5p	qRT-PCR	110	High expressions were associated with pathological tumor features of poor prognosis. miR-17-5p correlated with DFS only at early stages	[184]
miR-21	*In situ* hybridization	84	High miR-21 expressions were strongly associated with poor survival, more advanced TNM staging and poor therapeutic outcome	[90]
miR-29a	miRNA microarray, qRT-PCR	110	High expressions were associated with a longer DFS in CRC patients with stage II but not in stage I tumor	[61]
miR-34a-5p	qRT-PCR	205	The tissue expressions of miR-34a-5p was positively correlated with DFS. Moreover, expression of miR-34a-5p was an independent prognostic factor for CRC recurrence	[185]
miR-106a	qRT-PCR	110	Downregulation of miR-106a predicted shortened DFS and OS, independent of tumor stage	[184]
miR-132	miRNA microarray, qRT-PCR	28 (testing); 151 (validation)	Low expressions were associated with poor OS and occurrence of liver metastasis	[186]
miR-150	qRT-PCR, in situ hybridization	239	High expressions were associated with longer OS. Low expressions were associated with poor therapeutic outcome in patients treated with 5-FU-based chemotherapy with or without leucovorin, levamisole or cisplatin	[91]
miR-181a	qRT-PCR	162	High expressions were correlated with poor patient prognosis. Overexpression of miR-181a repressed the expression of the tumor suppressor (PTEN) at mRNA level	[187]
miR-181b	qRT-PCR	345	High expressions were correlated with poor survival in black patients with stage II CRC	[188]
miR-188-3p	Level 3 Illumina miRNASeq data were analyzed from TCGA database[c]	228	High expressions were associated with lower OS, higher tumor stage and indirectly with BRAF status	[99]
miR-195	qRT-PCR	85	Reduced expressions of miR-195 were correlated with occurrence of lymph node metastasis and advanced tumor stage	[189]
miR-199b	miRNA microarray and qRT-PCR	60	Higher level in metastatic CRC tissue compared with non-metastatic CRC tissue; low expressions were associated with longer OS	[190]
miR-203	microRNA microarray, qRT-PCR	197	High expressions were associated with more advanced TNM staging and poor survival	[90]
miR-215	qRT-PCR	34	High expressions were closely associated with poor OS	[191]
miR-218	qRT-PCR	63	High expressions were significantly associated with higher PFS, OS and response to 5-FU based chemotherapy	[192]
miR-320e	miRNA microarray and qRT-PCR	100	Elevated expressions were associated with poorer DFS and OS in stage III CRC patients	[93]
miR-429	qRT-PCR	116	High levels were correlated with OS; low levels were associated with favorable response to 5-FU-based chemotherapy	[193]
miR-494	qRT-PCR	104	High expressions were significantly associated with shorter DFS and OS. When used as a panel with 5 other miRNAs, the signature can distinguish early relapsed from non-early relapsed CRC.	[194]
miR-625-3p	qRT-PCR	94	High expressions were associated with higher OS, PFS and better response to treatment	[94]
3-miRNA signature (let-7i, miR-10b, miR-30b)	qRT-PCR	232	The addition of miR-30b to the 2-miRNA signature allowed the prediction of both distant metastasis and hepatic recurrence in patients with stage I-II CRC who did not receive adjuvant chemotherapy	[195]
A multi-RNA-based classifier (consisting of 12 mRNAs, 1 miRNA (miR-27a) and 1 lnc RNA)	mRNA, miRNA and lncRNA data were retrieved from the TCGA data portal	663	The classifier can divide patients into high and low risk groups with significantly different OS. Moreover, the classifier is not only independent of clinical features but also with a similar prognostic ability to the well-established TNM stage	[196]

OS: Overall survival ; DFS: Disease free survival; lnc RNA: Long non-coding RNA; TCGA : The Cancer Genome Atlas Project (http://tcga-data.nci.nih.gov/).
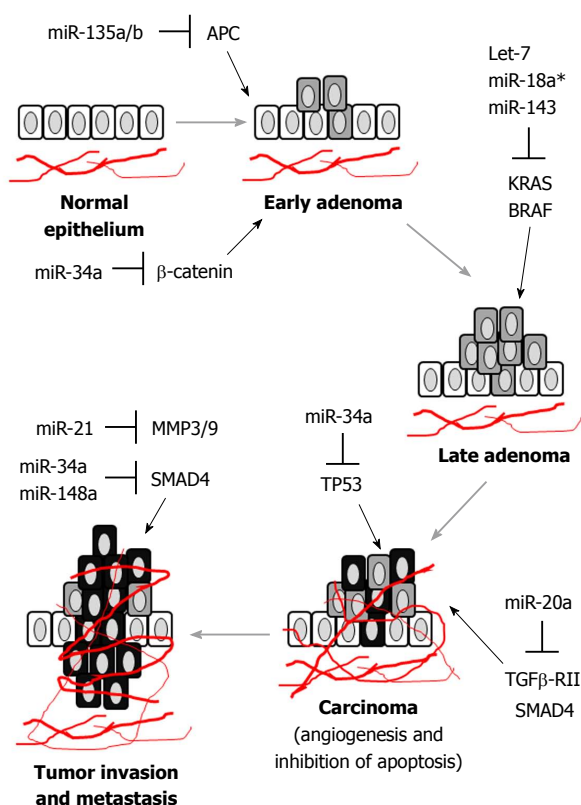
Figure 1 MicroRNA dysregulation and colorectal cancer progression. Altered expression of representative miRNAs are shown in different stages of CRC (in bold: Normal epithelium → early adenoma → late adenoma → carcinoma → metastasis). Upregulation or downregulation of miRNAs can affect signaling pathways and propel progression of CRC, leading to angiogenesis, cell invasion, metastasis and inhibition of apoptosis. APC: adenomatous polyposis coli; CRC: Colorectal cancer; KRAS: Kirsten rat sarcoma viral oncogene homolog; TP53: tumor protein p53.

detection in stool samples because they are protected in exosomes[72]. Moreover, fecal matter comes into direct contact with the lumen of colon and may include cells exfoliated from cancerous colonocytes. Molecular changes in CRC are more readily detected in the stool rather than in the blood[73]. Although fecal miRNAs were less extensively studied than circulatory miRNAs, the expression of numerous fecal miRNAs have been reported to be dysregulated in patients with CRC or advanced adenomas (Table 3). Recently, a panel of miRNAs isolated from the stool of CRC patients were found to differentiate not only CRC cases from healthy subjects but also differentiate TNM stages with high sensitivity and specificity[74]. Additionally, miR-135b was found to differentiate among different stages of CRC[75].

miRNAs in CRC-derived exosomes

Exosomes are a unique forms of extracellular vesicles, ranging from 30 nm to 100 nm in diameter, which are released into the extracellular space upon the fusion of multivesicular bodies with plasma membranes from diverse cell types. Tumor-derived exosomes are emerging as local and systemic intercellular mediators of oncogenic information through the horizontal transfer of

mRNAs, miRNAs, and protein during tumorigenesis[76]. In a recent proteome profiling study of exosomes derived from human primary and metastatic CRC cells, a selective enrichment of metastatic factors and signaling pathway components was observed[77]. High expression of exosomal miR-17-92a cluster was found to be associated with recurrence in late stage CRC patients[78]. Moreover, an elevated exosomal level of miR-19a was reported in the sera samples of CRC patients with poor prognosis[78]. Interestingly, a recent report has demonstrated an enrichment of miR-328 from CRC patients' plasma samples collected from colonic veins (i.e., mesenteric veins) when compared to peripheral vein plasma samples and that it was significantly correlated with the development of liver metastasis[79]. Table 4 summarizes a short list of miRNAs of prognostic value identified in exosomes released from CRC cells.

MIRNAS FOR STRATIFING RISK OF RECURRENCE IN STAGE II CRC PATIENTS

Adjuvant chemotherapy following surgery is adopted as the standard treatment to improve survival in stage III CRC patients[80]. Conversely, in stage II CRC patients, surgical resection of the primary tumor is highly effective without other concomitant treatment. Therefore, the use of adjuvant chemotherapy in all stage II CRC patients is debatable[81-83]. However, a notable sub-group of stage II CRC patients (~30%) develop tumor recurrence and have poor prognoses. At present, there are a few clinical parameters, including poorly differentiated histology, extramural venous invasion, intestinal obstruction and perforation, that are used as risk factors to identify high-risk patients for adjuvant chemotherapy. Since these clinical parameters are not specific, more reliable biomarkers are needed to identify the high-risk stage II CRC patients and initiate adjuvant chemotherapy to improve their survival. Likewise, other Stage II CRC patients with lower risk can be spared from the toxicity of conventional cytotoxic chemotherapeutic drugs. To this end, clinical trials have been initiated to investigate the use of miRNA to identify stage II CRC patients who may benefit from chemotherapy (including an on-going study being conducted in China (NCT02635087).

Schetter et al[84] showed that the combination of miR-21 expression in primary tumor tissues and inflammatory risk score was a better predictor of prognosis than either biomarker alone. Data from a large population-based study conducted on 520 stage II CRC patients showed that elevated miR-21 expression in tumor specimens correlated significantly with poor cancer-free survival[85]. Another six-miRNAs based classifiers (miR-20a-5p, miR-21-5p, miR-103a-3p, miR-106a-5p, miR-143-5p, and miR-215) were reported to be a promising predictive markers for tumor recurrence

Table 2 A list of representative circulating miRNAs of prognostic value in colorectal cancer patients

miRNA	Method of detection	Patient number	Observations and correlation with clinical outcome	Ref.
miR-21	qRT-PCR	102	Lower serum expressions were associated with higher local recurrence and mortality	[197]
miR-23b	qRT-PCR	96	CRC patients with low miR-23b expression in plasma exhibited a shorter recurrence-free survival and poorer overall survival rate	[198]
miR-96	TaqMan miRNA microarray	50 (screening); 234 (validation)	Elevated plasma levels were strongly correlated with shorter OS, especially in stage II and III CRC patients	[199]
miR-124-5p	qRT-PCR	71	Higher plasma levels were correlated with longer OS	[200]
miR-141	qRT-PCR	102	High plasma levels were significantly associated with stage IV colon cancer and poor prognosis	[70]
miR-148a	qRT-PCR	55	Lower levels were associated significantly with shorter DFS and poorer OS rates	[201]
miR-155	qRT-PCR	146	High serum levels correlated with poor PFS and OS	[202]
miR-183	qRT-PCR	118	High plasma levels were significantly associated with lymph node metastasis, distant metastasis, higher TNM staging, and tumor recurrence	[203]
miR-200b	TaqMan miRNA microarray	50 (screening); 234 (validation)	Elevated plasma levels were correlated with shorter OS, especially in stage II and III CRC patients	[199]
miR-200c	qRT-PCR	182 CRC patients and 24 controls	High serum expressions were strongly correlated with lymph node, distant metastasis, tumor recurrence and poor prognosis.	[204]
miR-203	qRT-PCR	144 (validation)	High serum levels were associated with poor survival and metastasis;	[205]
miR-218	qRT-PCR	189	Serum levels were significantly associated with TNM stage, lymph node metastasis and differentiation. Patients with low miR-218 serum level had shorter survival.	[206]
miR-221	qRT-PCR	103	Elevated plasma level is a significant prognostic factor for poor overall survival in CRC patients	[207]
miR-345	TaqMan miRNA microarray	138	High plasma levels were significantly associated with shorter PFS and lack of response to treatment with cetuximab and irinotecan	[108]
miR-885-5p	miRNA microarray, qRT-PCR	169	High serum expressions were highly correlated with poor prognosis, lymph node metastasis and distant metastasis	[208]
miR-1290	miRNA microarray, qRT-PCR	324	High serum levels were associated with lower OS, lower DFS and more advanced tumor stage	[209]
2-miRNA prognostic panel (miR-23a-3p & miR-376c-3p)	miRNA microarray	427	A prognostic panel consisting of miR-23a-3p and miR-376c-3p, independent of TNM stage, was established	[210]
miR-200 & miR-141	qRT-PCR	380	High serum levels of miR-200 and miR-141 were associated with higher propensity of CRC patients to develop liver metastasis	[211]
miR-122 & miR-200 family	miRNA microarray	543	Increased plasma miR-122 levels were associated with a "bad" prognostic subtype in metastatic CRC and a shorter relapse-free survival and overall survival for non-metastatic and metastatic CRC patients. Several members of the miR-200 family were associated with patients' prognosis and clinicopathological characteristics	[212]

OS : Overall survival; DFS: Disease free survival; PFS : Progression free survival; CRC: Colorectal cancer; TNM: Tumor, node, and metastasis.

in patients with stage II CRC[86]. More recently, the up-regulation of miR-181c in CRC specimens was also found to be associated with higher recurrence[87].

miRNAs for predicting response to chemotherapy
There has been intense research interest in the development of miRNA signature to predict response to anticancer treatment, thus allowing a more personalized approach for treating CRC with better efficacy and fewer adverse effects. To this end, a genome-wide miRNA profiling in twelve CRC cell lines has been performed to derive an *in vitro* signature of chemosensitivity[88]. Numerous studies have also reported the potential use of miRNAs as biomarkers in tissues and blood to predict response to the most common drug therapies (5-fluorouracil (5-FU)/oxaliplatin cytotoxic chemotherapy and EGFR targeted therapy) in CRC patients (Table 5).

Response to classical cytotoxic chemotherapy (5-FU/oxaliplatin) overexpression or downregulation of single miRNA in CRC tumor specimens
In 5-FU-based neoadjuvant chemotherapy setting

Table 3 A list of representative miRNAs identified in fecal samples from colorectal cancer patients that are of prognostic value

miRNA	Method of detection	Patient number	Observations and correlation with clinical outcome	Ref.
miR-135b	qRT-PCR	424	miR-135b showed a significant increasing trend across the adenoma to cancer sequence. miR-135b level may be used to differentiate between different stages of CRC. Stool miR-135b level dropped significantly upon removal of CRC or advanced adenoma.	[75]
miR-19-b-3p, miR-20a-5p, miR-21-3p, miR-92a-3p, miR-141	qRT-PCR	20	Expression levels of three out of the five overexpressing miRNAs returned to values comparable to normal controls after curative surgery; this was correlated with the	[213]
12 upregulated and 8 downregulated miRNA panel	miRNA microarray, qRT-PCR	60	A panel of 12 upregulated miRNAs (miR-7, miR-17, miR-20a, miR-21, miR-92a, miR-96, miR-106a, miR-134, miR-183, miR-196a, miR-199a-3p, miR-214) and 8 downregulated miRNAs (miR-9, miR-29b, miR-127-5p, miR-138, miR-143, miR-146a, miR-222, miR-938) were found to differentiate not only CRC cases from healthy subjects but also differentiate TNM stages with high sensitivity and specificity.	[74]

Table 4 A list of representative miRNAs isolated from exosomes in biological samples from colorectal cancer patients or cell lines that are of prognostic value

miRNA	Method of detection	Type of samples	Tumor stage	Ref.
miR-17-92a	miRNA microarray	Tumor specimens	IV	[78]
miR-19a	miRNA microarray	Tumor specimens	IV	[78]
let-7a	qRT-PCR	Serum	I, II, IIIa, IIIb, IV	[214]
miR-21	qRT-PCR and miRNA microarray	Serum; plasma	I, II, IIIa, IIIb, IV	[214]
miR-23a	qRT-PCR	Serum	I, II, IIIa, IIIb, IV	[214]
miR-150	qRT-PCR	Serum	I, II, IIIa, IIIb, IV	[214]
miR-203	qRT-PCR	Serum	I, II, III, IV	[215]
miR-223	qRT-PCR	Serum	I, II, IIIa, IIIb, IV	[214]
miR-1246	qRT-PCR	Serum	I, II, IIIa, IIIb, IV	[214]
miR-1229	qRT-PCR	Serum	I, II, IIIa, IIIb, IV	[214]
miR-548c-5p	miRNA microarray and qRT-PCR	Serum (downregulated)	I, II, IIIa, IIIb, IV	[216]
miR-638	miRNA microarray and qRT-PCR	Serum (downregulated)	I, II, IIIa, IIIb, IV	[216]
miR-5787	miRNA microarray and qRT-PCR	Serum (downregulated)	I, II, IIIa, IIIb, IV	[216]
miR-8075	miRNA microarray and qRT-PCR	Serum (downregulated)	I, II, IIIa, IIIb, IV	[216]
miR-6869-5p	miRNA microarray and qRT-PCR	Serum (downregulated)	I, II, IIIa, IIIb, IV	[216]
miR-486-5p	miRNA microarray and qRT-PCR	Serum (upregulated)	I, II, IIIa, IIIb, IV	[216]
miR-3180-5p	miRNA microarray and qRT-PCR	Serum (upregulated)	I, II, IIIa, IIIb, IV	[216]
miR-96-5p and miR-149	qRT-PCR	Plasma	III	[217]
miR-100	qRT-PCR	Cell lines (DKO-1, Dks-8, DLD-1)		[218]
miR-192	qRT-PCR	Cell lines (HCT-15, SW480, WiDr)		[219]
miR-210	qRT-PCR	Cell line (HCT-8)		[220]
miR-221	qRT-PCR	Cell lines (HCT-15, SW480, WiDr)		[219]
miR-379	qRT-PCR	Cell lines (HCT-116, HT-29)		[221]

to shrink the tumor size before surgery, low tumoral expression of miR-21 was found to be predictive of pathological response to the drug in locally advanced CRC patients[89]. In another study performing miRNA expression profiling of CRC, along with their paired non-cancerous tissues, high miR-21 expression in the tumors were found to associate with poor therapeutic response to 5-FU-based adjuvant chemotherapy as an add-on treatment[90]. Also predictively, low miR-148a, low miR-150 or high miR-320e level in tumor specimens were found to be associated with poor response to adjuvant 5-FU/oxaliplatin (FOLFOX) regimen in several other studies[91-93]. Interestingly, in a recent study on

responsive and non-responsive metastatic CRC patients receiving XELOX/FOLFOX regimen, high expression of mR-625-3p in the tumor was found to be associated with poor response to the drug but was not related to disease recurrence[94].

Signature of miRNAs panel in tumor specimens

While the increased/decreased expression of a single miRNA may predict response to chemotherapy as described above, the use of a panel of miRNAs demonstrating a signature may be more reliable and specific. In a recent study on fluoropyrimidine-based chemotherapy, the responders were found to exhibit high tumoral levels

Table 5 MiRNAs in tumor specimens and plasma/serum samples reported to predict therapeutic response in colorectal cancer patients

miRNA	Treatment regimen	Detection method	Expression that suggests inadequate response	Patient number	Ref.
Tumor specimens					
Let-7	Irinotecan, cetuximab	qRT-PCR	Low	59	[102]
miR-7	Cetuximab	qRT-PCR	Low	105	[97]
miR-21	5-FU	qRT-PCR	High	84	[90]
miR-21	5-FU	qRT-PCR	High	67	[89]
miR-21-5p	5-FU + radiation	Microarray	Low	27	[222]
miR-31-3p	Anti-EGFR	Microarray, qRT-PCR	High	33	[98]
miR-31-5p	Anti-EGFR	qRT-PCR	High	102	[100]
miR-126	Capecitabine, oxaliplatin	qRT-PCR, ISH	Low	89	[104]
miR-143	Capecitabine, oxaliplatin, anti-EGFR	Microarray, qRT-PCR	High	34	[103]
miR-146b-3p	Cetuximab	qRT-PCR	High	25	[223]
miR-148a	5-FU	qRT-PCR, ISH	Low	273	[92]
miR-150	5-FU	qRT-PCR, ISH	Low	239	[91]
miR-181a	Anti-EGFR	qRT-PCR	Low	80	[99]
miR-200 family	Fluoropyrimidine	qRT-PCR	Low	127	[103]
miR-200b	Capecitabine, oxaliplatin, anti-EGFR	Microarray, qRT-PCR	Low	34	[103]
miR-320e	5-FU, oxaliplatin	Microarray, qRT-PCR	High	100	[93]
miR-455-5p	Capecitabine, oxaliplatin, bevacizumab	qRT-PCR, ISH	High	212	[224]
miR-486-5p	Cetuximab	qRT-PCR	High	25	[223]
miR-519c	5-FU, irinotecan	qRT-PCR	Low	26	[153]
miR-592	Anti-EGFR	Microarray	Low	33	[98]
miR-625-3p	Capecitabine, oxaliplatin	Microarray, qRT-PCR	High	94	[94]
miR-664-3p	Capecitabine, oxaliplatin, bevacizumab	qRT-PCR, ISH	Low	212	[224]
Let-7c, miR-99a, miR-125b	Anti-EGFR	Microarray, qRT-PCR	Low	74	[101]
miR-1274b, miR-720	Capecitabine, oxaliplatin, radiation	Microarray, qRT-PCR	High	38	[96]
miR-107, miR-99a-3p	Fluoropyrimidine	Microarray, qRT-PCR	Low	39	[225]
miR-215, miR-190b, miR-29b-2	5-FU, radiotherapy	Microarray, qRT-PCR	High	20	[95]
Let-7e, miR-196, miR-450a, miR-450b-5p, miR-99a	5-FU, radiotherapy	Microarray, qRT-PCR	Low	20	[95]
miR-17-3p, miR-193b-5p, miR-204-5p, miR-501-5p, miR-545-3p, miR-592, miR-644-3p, miR-15a-5p, miR-196b-5p, miR-552	First-line capecitabine and oxaliplatin with or without bevacizumab	qRT-PCR, ISH	Low	212 (screening); 121 (validation)	[224]
miR-1183, miR-622. miR-765, miR-1471, miR-125a-3p, miR-1224-5p, miR-188-5p, miR-483-5p, miR-671-5p, miR-1909	Capectabine, oxaliplatin, radiation	Microarray, qRT-PCR	Low	38	[96]
Blood					
miR-19a	FOLFOXa	Microarray, qRT-PCR	High	72	[226]
miR-126	Bevacizumab		High		[104]
miR-143	Oxaliplatin	qRT-PCR	Low	62	[227]
miR-155	Leucovorin, cetuximab, 5-FU	qRT-PCR	High	15	[107]
miR-345	Cetuximab, irinotecan	Microarray, qRT-PCR	High	138	[108]
miR-1914*	Capecitabine + oxaliplatin	qRT-PCR	Low	49	[228]
miR-106a, miR-130b, miR-484	5-FU, oxaliplatin	qRT-PCR	High	150	[105]
miR-20a, miR-130, miR-145, miR-216, miR-372	FOLFOX	Microarray, qRT-PCR	High	40	[106]

FOLFOX: 5-FU + oxaliplatin + leucovorin; 5-FU: 5-fluorouracil; ISH: *In situ* hybridization; Anti-EGFR: Anti-EGFR targeted monoclonal antibody therapy; EGFR: Epidermal growth factor receptor.

of let-7e, miR-99a*, miR196b, miR450a and miR450b-5p whereas the non-responders were found to have high expression of miR-29b-2*, miR-196b and miR-215[95]. Another set of thirteen miRNAs, exhibiting a unique signature with 11 upregulated miRNAs (miR-125a-3p, miR-188-5p, miR-483-5p, miR-622, miR-630, miR-671-5p, miR-765; miR-1183, miR-1224-5p, miR-1471, and miR-1909*) and 2 downregulated miRNAs (miR-720 and miR-1274b) were shown to be strongly associated with complete pathological response to neoadjuvant

capecitabine and oxaliplatin (XELOX) regimen in CRC patients[96].

Response to anti-EGFR/anti-VEGF targeted therapy

There has been intense research effort to identify miRNAs predictive of a favorable response to anti-EGFR monoclonal antibody. Consistent with the fact that miR-7 targets EGFR, reduced miR-7 expression was linked to poor prognosis of anti-EGFR therapy[97]. The efficacy of anti-EGFR therapy is related to the mutation status of KRAS/BRAF. Patients bearing wild-type KRAS/BRAF are normally considered candidates for anti-EGFR therapy. Therefore, numerous studies have been carried out with an aim to identify miRNAs that predict success with EGFR inhibition with wild-type KRAS. In metastatic CRC patients bearing wild-type KRAS/BRAF, high expression of miR-31[98], low expression of miR-592[98], or low expression of miR-181a[99] were found to be strongly associated with disease progression and short progression-free survival after treatment with anti-EGFR therapy (cetuximab or panitumab). To this end, miR-31-5p has been reported to regulate BRAF activation and thus the downstream signaling pathway of EGFR[100]. Conversely, miR-181a has been reported to regulate β-catenin expression[99]. Apart from individual miRNAs, the high-intensity signature of the cluster let-7c/miR-99a/miR-125b was found to be associated with favorable response (longer progression-free survival and overall survival) to cetuximab or panitumumab in metastatic CRC patients bearing wild-type *KRAS*[101].

Drug resistance mediated by *KRAS* mutations is severely hindering the clinical efficacy of anti-EGFR therapy in CRC because *KRAS* mutations drive the downstream MAPK and Akt signaling independent of EGFR. However, treatment with anti-EGFR therapy is still feasible, and a few miRNAs have been reported to predict favorable responses in metastatic CRC patients bearing *KRAS* mutations. To this end, let-7 is the most extensively studied miRNA as predictive biomarker for anti-EGFR therapy in patients with *KRAS* mutations[102]. Let-7 downregulates KRAS by directly binding to its mRNA 3'UTR. Interestingly, in patients with *KRAS* mutations, high levels of let-7 were shown to be significantly associated with better survival following cetuximab treatment[102]. Thus, let-7 level may be used to select subgroup of CRC patients with *KRAS* mutations to benefit from anti-EGFR therapy. In another study investigating *KRAS*-mutated metastatic CRC tumors, low miR-143 and high miR-200b expression in the tumor were found to be associated with better progression-free survival after treatment with combination of XELOX, cetuximab and bevacizumab[103].

The combination of anti-VEGF monoclonal antibody (bevacizumab) and cytotoxic chemotherapy has been approved for treating metastatic CRC. The predictive value of circulating miR-126 in treatment response to first-line chemotherapy combined with bevacizumab has

been demonstrated in patients with metastatic CRC[104]. While non-responding patients had a remarkable increase in circulating miR-126 level, responding patients exhibited a mild decrease in circulating miR-126.

CIRCULATING MIRNAS AS NON-INVASIVE BIOMARKERS OF DRUG RESPONSE IN CRC

Besides serving as prognostic markers, circulating miRNAs can also be used as non-invasive and convenient biomarkers of drug response to classical cytotoxic drugs and EGFR-targeted therapy in CRC patients.

Pre-treatment level of plasma miRNAs have been shown to predict treatment response to 5-FU/oxaliplatin in metastatic colorectal cancer patients[105]. By measuring the expression profile of plasma miRNAs in metastatic CRC patients before and after four cycles of 5-FU/oxaliplatin chemotherapy, a set of three miRNAs (miR-106a, miR-130b, and miR-484) were found to be significantly upregulated in non-responders[105]. In another study using Taqman low-density array to evaluate the expression profile of serum miRNAs in chemotherapy responsive versus resistant CRC patients by unsupervised cluster analysis, a set of five serum miRNAs (miR-20a, miR-130, miR-145, miR-216, and miR-372) was found to predict response to classical chemotherapy and it may guide the selection of the chemotherapy regimen[106]. Recent studies have also shown that elevated levels of serum miR-155[107] and miR-345[108], respectively, were associated with inadequate response to 5-FU/leucovorin/cetuximab and irinotecan/cetuximab regimens in metastatic CRC patients.

POLYMORPHISMS IN MIRNA BINDING SITES PREDICT CRC PROGNOSIS AND TREATMENT RESPONSE

Single nucleotide polymorphisms (SNPs) located in the 3' UTR of miRNA target genes have been shown to affect miRNA-mRNA interaction and thus expression of target genes[109,110]. While several studies have reported the correlation between miRNA SNPs and risk of developing CRC[110,111], only a few studies have been conducted to investigate the use of miRNA SNPs to predict clinical outcomes of cancer therapy.

Using genome-wide approach, two SNPs rs1051424 and rs11704 were identified in the putative miRNA target sites of the 3'UTR of RPS6KB1 and ZNF839[112]. Importantly, overall survival was found to be inversely proportional to the occurrence of the two SNPs in CRC patients[112]. The SNPs resulted in the loss of the miRNA binding sites on both RPS6KB1 and ZNF839, thereby enabling the upregulation of the two genes. Activated RPS6KB1 is known to phosphorylate ribosomal protein

S6 to increase protein synthesis and to promote cancer cell survival[113]. On the other hand, ZNF839 is not well characterized but reportedly involved in humoral immune response to cancer[114].

Similar miRNA SNP-mRNA interaction has also been shown to predict therapeutic response to cancer treatment in CRC. While it is well known that KRAS mutations predict resistance to anti-EGFR monoclonal antibodies treatment in CRC, the predictive marker for therapeutic response in CRC patients bearing wild-type *KRAS* is less studied. In a phase II study, the occurrence of a SNP in the let-7 miRNA complementary site of wild-type *KRAS* 3'UTR was found to correlate with the objective response rate to cetuximab monotherapy in metastatic CRC patients[115].

MIRNAS DEMONSTRATED TO BE THERAPEUTIC TAGETS IN CRC

The development of miRNA-based therapy for cancer treatment is based on the premise that aberrantly expressed miRNAs play crucial role in the development of cancer and therapeutic response to anticancer drugs. Therefore, correcting the miRNA deficiency or restoring the miRNA function could be used as novel strategy in cancer treatment[116,117]. Key miRNAs that have been studied are described below.

miR-21 and tumor invasion
Overexpression of miR-21 was associated with tumor invasion of CRC by promoting nuclear translocation of β-catenin[118]. Interestingly, CRC patients bearing *adenomatous polyposis coli* (*APC*) mutated tumor who have high levels of serum miR-21 were found to have poor prognosis, but this correlation was not observed in CRC patients bearing *APC*-wild type tumor. Mechanistically, miR-21 targets PDCD4, which inhibits cancer transformation and invasion[119].

Furthermore, miR-21 was also shown to confer resistance to 5-FU chemotherapy by downregulation of human MutS homolog 2 (*MSH2*)[120]. Overexpression of miR-21 was found to suppress the G2/M arrest and apoptosis induced by 5-FU. To this end, a miR-21 antisense inhibitor against hepatocellular carcinoma is under development by Regulus Therapeutics[121].

miR-34a and tumor suppressor p53
miR-34a is one of the most important downstream molecules of p53 that is transcriptionally activated to exert the tumor suppressive effect through cell cycle arrest and induction of apoptosis[122,123]. However, p53 mutation is observed in 50%-75% of CRC[3]. Downregulation of miR-34a is commonly observed in CRC. It has been reported that miR-34a expression was significantly decreased in CRC patients versus healthy individuals, suggesting that miR-34a could be a useful biomarker and therapeutic target in CRC[124,125]. Recently, it has been shown that p53-dependent expression of miR-34a blocks the IL-6R/STAT3/miR-34 feedback loop and subsequently inhibit tumor progression in CRC[126]. Since activation of IL-6R and STAT3 promote cancer proliferation, the overexpression/restoration of miR-34a may develop into a new treatment strategy for CRC.

miR-101 and WNT/β-catenin pathway
More than 60% of CRC cases are known to carry inactivating *APC* gene mutations, which lead to stimulation of the Wnt/β-catenin pathway to drive tumor initiation and recurrence[3]. Besides this extensively studied APC gene-mediated mechanism, miRNAs have also been shown to regulate the Wnt pathway in CRC. Downregulation of miR-101 was associated with activation of Wnt/β-catenin pathway[127]. Interestingly, overexpression of miR-101 in CRC was found to impair β-catenin nuclear localization and inhibit cancer stem cells-related genes[127]. Therefore, pharmacological restoration of miR-101 may represent a novel strategy to prevent recurrence of CRC.

miR-135B and downregulation of APC in CRC
miR-135a/b has been shown to be upregulated in colorectal adenomas and carcinomas, which correlates well with low APC expression in the tumor[128]. MiR-135a/b was found to target the 3'UTR of APC to suppress the expression of *APC* gene, and thus activating the downstream Wnt pathway to promote tumor transformation and progression[129]. To this end, miR-135b up-regulation was also found to be common in sporadic and inflammatory bowel disease-associated human CRCs; similarly, increased miR-135b expression was correlated with advanced tumor stages and poor clinical prognosis[129]. Importantly, inhibition of miR-135b, in CRC mouse xenograft models, was able to inhibit tumor growth by regulating genes involved in proliferation, invasion and apoptosis.

miR-143/145 as tumor suppressors in CRC
miR-143 and miR-145 were first identified as potential tumor suppressors in an analysis investigating the alteration of microRNAs expression from adenomatous to carcinoma stages in CRC[8]. Consistent with this finding, the upregulation of miR143/145 was observed in tumor tissues from advanced rectal cancer patients demonstrating favorable response to neoadjuvant chemoradiotherapy[130]. Mechanistically, miR-143 was found to bind directly to and suppress KRAS, DNMT3A and ERK5 whereas miR-145 was shown to target IRS-1, c-Myc, FLI1, IRS-1, STAT1 and YES1 to suppress tumor growth[131]. Forced expression of miR-143 and miR-145 have been shown to inhibit proliferation of CRC cell lines *in vitro* and *in vivo*[132].

MIRNAS REGULATING THE EGFR SIGNALING PATHWAY

The crucial role played by EGFR signaling in the survival

of numerous cancer types, including CRC, has led to the approval of anti-EGFR targeted therapy, including tyrosine kinase inhibitors and monoclonal antibodies for treating CRC. However, drug resistance to these targeted agents is severely hindering their clinical efficacy. To this end, the activation of PI3K and mutation of KRAS, two key signaling pathways downstream of EGFR, have been extensively studied as mechanisms leading to the drug resistance to anti-EGFR therapy[133]. In recent years, increasing evidence has suggested that aberrant expression of miRNAs are affecting EGFR signaling and are causing drug resistance to anti-EGFR therapy.

Low expression of miR-30b was observed in CRC tumor specimens, which was significantly associated with advanced TNM staging and poor prognosis[24]. Forced expression of miR-30b was found to promote G1 arrest and induce apoptosis by targeting PIK3CD and BCL2, thereby inhibiting CRC cell growth *in vitro* and *in vivo*. On the other hand, miR-126 has been shown to inhibit PI3K signaling by targeting the p85b regulatory subunit responsible for PI3K stabilization[19].

Activating mutation of KRAS is known to drive resistance to anti-EGFR therapy by bypassing the inhibited EGFR. To this end, let-7 has been shown to post-transcriptionally downregulate KRAS and administration of let-7 to CRC animal models expressing activating KRAS mutations was shown to inhibit tumor growth[134]. More importantly, in CRC patients bearing mutant KRAS tumor who received salvage cetuximab plus irinotecan, higher let-7 level was found to be significantly associated with better survival outcomes[102]. miR-4689 was found to directly target KRAS and AKT1, therefore activation of the KRAS pathway may thus be mediated by downregulation of miR-4689. Forced expression of miR-4689 has been reported to inhibit CRC cancer growth and induce apoptosis both *in vitro* and *in vivo*[135].

The tumor suppressor PTEN (phosphatase and tensin homolog) plays an important role in regulating the PI3K/Akt pathway. Overexpression of miR-17-5p has been shown to promote drug resistance and tumor metastasis of CRC by downregulating PTEN[136]. Similarly, miR-32 was found to directly repress PTEN to regulate tumorigenesis of CRC[137].

MIRNAS REGULATING EMT

EMT is a complex process controlled by a number of transcriptional regulators and signaling pathways. In CRC, EMT is associated with a more malignant phenotype and metastasis. Recently, miRNAs have been suggested to be involved in the acquisition of cancer stem cell-like properties by regulating EMT. To this end, TGF-β is a major inducer of EMT by activating Smad signaling pathway. The miR-200 family have been reported to target TGF-β2. In particular, miR-

200c was found to be significantly upregulated in tumor samples from patients with metastatic colon cancer and also colon cancer cell lines[37], which has been correlated with reduced expression of its direct target (ZEB1) to inhibit EMT[37,138,139].

miR-130a/301a/454 family of microRNAs have also been shown to regulate TGF-β signaling by inhibiting SMAD4[140]. Overexpression of these miRNAs was reported to increase cell proliferation and migration in colon cancer cell lines (HCT116 and SW480)[140]. Similarly, miR-1269a was also found to promote EMT and metastasis in CRC cells *in vitro* and *in vivo*[141]. Importantly, the overexpression of miR-1269a in tumor specimens from patients with late-stage CRC was associated with relapse and metastasis[141].

MIRNAS AS REGULATORS OF DRUG RESISTANCE IN CRC

While surgical resection of the primary tumor is the treatment of choice for most CRC patients, chemotherapy is commonly used as both neoadjuvant and adjuvant therapy to reduce the tumor load and to prevent recurrence and metastasis, respectively. Commonly used chemotherapeutic agents include 5-FU, oxaliplatin, irinotecan, and the anti-EGFR/VEGF monoclonal antibodies[142]. Drug resistance to these anticancer drugs pose a major obstacle for effective chemotherapy[143]. To this end, miRNAs have been found to play an important role in the induction of chemoresistance and they also represent emerging prognostic markers to indicate treatment success[144-146] (Table 6).

miRNAs mediating resistance to 5-FU

5-FU is the most commonly used first-line chemotherapeutic drug for CRC. Hypoxia inside CRC tumors has been shown to reduce drug sensitivity to 5-FU by upregulating two hypoxia-responsive miR-21 and miR-30d, which altered amino acid metabolism. Importantly, treatment with miR-21 and miR-30d antagonists were able to sensitize hypoxic and resistant CRC cells to 5-FU[147]. Reduced miR-34a expression in CRC cell line was also shown to mediate resistance to 5-FU. Ectopic expression of miR-34a was able to reverse the resistance by downregulating Sirt1 and E2F3[148]. It has been demonstrated that high expression of miR-23a could trigger 5-FU resistance by inhibiting the APAF-1/caspase-9 apoptotic pathways in CRC cell lines[149]. While miR-494 expression was found to be reduced in drug-resistant cell lines, forced expression of miR-494 was shown to potentiate the anticancer effect of 5-FU by targeting DPYD[150]. On the other hand, overexpression of miR-587 has been observed in CRC cells resistant to 5-FU *in vitro* and *in vivo*[151], which is mediated by downregulation of PPP2R1B and thus increased Akt phosphorylation and elevated XIAP

Table 6 A list of representative miRNAs involved in drug resistance to colorectal cancer therapy

miRNA	Drug(s) affected	Known molecular target(s) in CRC	Ref.
Overexpression of miRNA causing drug resistance to conventional chemotherapeutic drugs			
Let-7g	S-1 (Tegafur/gimeracil/oteracil)	Cyclin D, c-myc, E2F, RAS	[229]
miR-10b	5-FU	BIM	[230]
miR-19b	5-FU	MYBL2	[231]
miR-20a	5-FU, oxaliplatin	BNIP2	[170]
miR-21	5-FU	hMSH2 and hMSH6	[120]
miR-23a	5-FU	APAF-1	[149]
miR-31	5-FU	-	[232]
miR-140	5-FU	HDAC4	[233]
miR-148a	5-FU, oxaliplatin	-	[92]
miR-181b	S-1, 5-FU	Cyclin D, c-myc, E2F, RAS	[229]
miR-192/miR-215	5-FU	DHFR	[234,235]
miR-195	5-FU	WEE1	[236]
miR-203	Oxaliplatin	ATM	[154]
miR-224	5-FU	-	[237]
miR-520g	5-FU, oxaliplatin	p21	[238]
miR-587	5-FU	PPP2R1B	[151]
miR-625-3p	Oxaliplatin	-	[94]
Downregulation of miRNA causing drug resistance to conventional chemotherapeutic drugs			
miR-34a	5-FU, oxaliplatin	SIRT1, KIT, LDHA, TGF-β, SMAD4	[239-241]
miR-139-5p	5-FU	NOTCH-1	[242]
miR-153	Oxaliplatin	FOXO3	[243]
miR-194	Oxaliplatin, irinotecan	HMGA2	[244]
miR-200 family	5-FU	EMT-related genes	[245]
miR-200b-3p	Oxaliplatin	PRDX2	[246]
miR-203	5-FU	TYMS	[247]
miR-218	FOLFOX	EZH2	[248]
miR-761	5-FU	FOXM1	[249]
miR-1915	Oxaliplatin	BCL2	[155]
miR-141/miR-200c	Oxaliplatin	ZEB1	[250]
miR-18a*/miR-4802	5-FU, capecitabine	ATG7, ULK1	[251]
Overexpression of miRNA causing drug resistance to molecular targeted drugs			
miR-31	Cetuximab	-	[252]
miR-126	Bevacizumab	VEGF	[104]
miR-100/miR-125b	Cetuximab	Wnt/β-catenin negative regulators	[253]
Downregulation of miRNA causing drug resistance to molecular targeted drugs			
Let-7 family	Cetuximab, panitumumab	KRAS	[115,223]
miR-7	Cetuximab	EGFR, ERK1/2, AKT	[97]

EGFR: Epidermal growth factor receptor; CRC: Colorectal cancer.

expression to inhibit apoptosis.

Overexpression of the multidrug resistance transporter ABCG2 has been shown to cause resistance to 5-FU. Our research team has previously demonstrated the specific targeting of ABCG2 by miR-519c[152]. Interestingly, we revealed the shortening of the ABCG2 3'UTR in several ABCG2-overexpressing resistant cell lines, which removes the miR-519c binding site and has a repressive effect on mRNA stability and translocation blockadeallowing ABCG2 overexpression without a change in miR-519c expression to mediate drug resistance[152]. We further validated the effect of ABCG2 dysregulation on CRC sensitivity to 5-FU-based chemotherapy using pairs of archival tissue blocks of CRC tumors and their matched non-cancerous colon epithelial cells from CRC patients[153]. In the unresponsive tumor, high ABCG2 expression was found to be associated with the concomitant overexpression of a mRNA binding protein HuR but a low expression of miR-519c because miR-519c was known to target both HuR and ABCG2[153].

miRNAs mediating resistance to oxaliplatin

Oxaliplatin is a key platinum-based anticancer drug in combination regimens for CRC treatment. High expression of miR-203 has been shown to mediate oxaliplatin resistance by suppressing the key DNA double-strand break sensor ATM[154]. Likewise, high expression of miR-625-3p was associated with poor response to first-line treatment with oxaliplatin in metastatic CRC[94]. Moreover, forced expression of miR-1915 in an oxaliplatin-selected multidrug resistant cell line HCT116/L-OHP was found to induce apoptosis by inhibiting Bcl-2[155].

miRNAs mediating resistance to irinotecan

Irinotecan is another key cytotoxic chemotherapeutic drug in the FOLFIRI regimen (5-FU + leucovorin +

Table 7 miRNA therapeutics investigated in animal models of colorectal cancer				
miRNA	Animal model	Restoring or suppressing miRNA function	Therapeutic outcome	Ref.
miR-34a	Transgenic mice	Restoring	Anti-tumor	[126]
miR-135b	Xenotranplantation of tumor-derived organoids to mice	Suppression (antisense)	Anti-tumor	[129]
miR-143	Mice xenograft	Restoring	Anti-tumor	[132]
miR-145	Mice xenograft	Restoring	Negative effect	[132]
miR-4689	Mice xenograft	Restoring (mimic)	Anti-tumor	[135]

irinotecan) for metastatic CRC. In colonospheres exhibiting cancer stem cell properties and chemoresistance from CRC cell lines, miR-451 was found to be remarkably downregulated[156]. Restoration of miR-451 in the colonospheres was found to specifically reverse drug resistance to irinotecan by decreasing the expression of the drug efflux ATP-binding cassette drug transporter ABCB1[156].

miRNAs mediating resistance to targeted drugs for CRC
Clinical efficacy with the anti-EGFR or anti-VEGF monoclonal antibodies in CRC is known to be severely hindered by drug resistance due to numerous mechanisms. Downregulation of miR-216b has been reported to mediate adapted autophagy (through upregulation of Beclin-1) in CRC cell lines to mediate resistance to anti-EGFR therapy[157]. Strategies to restore miR-216b or inhibit autophagy are thus proposed to improve the efficacy of anti-EGFR therapy in CRC. Conversely, the reduced expression of a tumor suppressor miR-7, which targets EGFR and RAF-1, was observed in CRC tumor specimens from patients unresponsive to cetuximab[97]. Reduced expression of miR-181a in tumor specimens from CRC patients was also found to correlate with poor response to EGFR antibodies[99].

MODULATING MIRNAS FOR TREATMENT OF CRC

Modulation of miRNA expressions and/or miRNAs-regulated pathways may be used as a therapeutic strategy to potentiate the cytotoxic effect from other anticancer drugs in CRC[158]. Guidelines have been proposed for the investigation and evaluation of potential microRNA-based therapeutics for cancer therapy[159]. Briefly, the up- or downregulation of miRNAs have to be established in appropriate cancer models. Gain/loss of function studies are then performed *in vitro* and in animal models. After the potential miRNA targets have been identified, an efficient delivery system has to be available to administer the miRNA modulating modalities for pharmacological, pharmacokinetic and pharmacodynamic studies. Finally, clinical trials will be conducted to evaluate the efficacy and safety of the potential miRNA therapeutics in patients. Only a few miRNA modulating systems have been investigated *in*

vivo, which are summarized in Table 7.

RESTORING TUMOR SUPPRESSIVE MIRNAS

Downregulation of tumor-suppressive miRNAs are known to contribute to cancer development. Strategies have been devised to effectively restore the function of these "lost" miRNAs using synthetic miRNA-like molecules, including miRNA mimics and miRNAs encoded in expression vectors. MiRNA mimics are short and chemically modified (2'-O-methoxy) RNA duplexes, which are processed into single-strand form intracellularly and loaded into RNA-induced silencing complex (RISC) to mediate the target mRNA degradation and/or post-translational blockade[160,161]. This approach has been investigated in cell models and orthotopic mice models of colorectal and hepatocellular carcinomas and hepatic metastasis from primary CRC to restore the expression of downregulated miR-26a, miR-33a, miR-34a and miR-145[159,162-164]. The restoration of these tumor suppressor miRNAs was found to inhibit cancer cell proliferation, induce apoptosis, shrink tumor size and prolong animal survival.

miR-34 is remarkably downregulated in a number of different cancer types including CRC[165]. As discussed above, miR-34 has been shown to regulate multiple oncogenic pathways. Thus, the restoration of miR-34 may represent a novel therapeutic approach to treat cancer. To this end, a first-in-human phase I clinical trial (NCT01829971) has been conducted to investigate the safety, pharmacokinetics and clinical activity of a liposomal formulation of miR-34 mimic (known as MRX34) in patients with advanced solid tumors including CRC[166]. While MRX34 was tolerated and displayed antitumor activity in a subset of patients with refractory advanced solid tumors[166], the sponsoring company (Mirna Therapeutic, Inc.) has voluntarily halted the enrollment and dosing of MRX34 in clinical study in September 2016 following multiple immune-related severe adverse events reported in patients dosed with MRX34.

INHIBITING ONCOGENIC MIRNAS

Different approaches have been developed to inhibit mature miRNAs, which include antisense oligonucleotides (also known as ASOs, anti-miRs, or antagomirs), miRNA

masking, miRNA sponges, and anti-miR peptides. ASOs are single-stranded oligonucleotides that are complementary to the target miRNA[167]. The miRNAs bind to the RISC complexes, which subsequently block the interaction of miRNAs with their endogenous mRNA targets. This strategy has been evaluated in cell culture and mice models of CRC by targeting overexpressed oncogenic miRNAs. Specific inhibition of miR-135b has been achieved in human colon cancer SW480 cells to inhibit cell proliferation and to induce apoptosis[129] and in HCT116 cells to suppress cell migration[168]. Other oncogenic miRNAs, including miR-20a, miR-21, miR-95, and miR-675, have also been inhibited by similar approaches in human CRC cell lines[120,169-171]. ASOs are often chemically modified by conjugation to cholesterol to avoid cleavage by RISC nuclease and to increase their stability. Locked nucleic acids (LNAs) are nucleic acid analogs in which the ribose ring is "locked" with a methylene bridge connecting the 2′-O atom with the 4′-C atom[172]. Modification by LNAs is another approach used to enhance stability of ASOs, which also increases the binding affinity to the target miRNA[173]. LNA-containing oligonucleotides also have excellent mismatch discrimination capabilities[174], thus avoiding potential off-target secondary effects. LNA-anti-miR-21 was an ASO developed by the LNA technology[175]. It has been shown to inhibit cancer growth and invasion in a colon cancer cell line LS174T[175]. On the other hand, miRNA masking refers to the use of single-stranded 2′-O-methyl modified oligonucleotides complementary to miRNA binding sites in the 3′UTR of the target mRNAs to interfere with specific miRNA-mRNA interaction[176]. A miR-34a sponge construct, encoding a decoy mRNA containing multiple tandem miR-34a binding sites, has been shown to inhibit self-renewal of colon cancer stem cells *in vitro*[177]. Anti-miR peptides are peptide nucleic acids (PNAs), modified from DNA or RNA, with the sugar backbone replaced by a peptide-like backbone consisting of N-(2-aminoethyl)glycine units. Aside from being more stable and able to be administered systematically with low toxicity, PNAs have been shown to bind to target miRNA more tightly than equivalent oligonucleotides[178].

USE OF SMALL MOLECULE MODULATORS OF MIRNA EXPRESSION

Besides making use of pharmaceutical carriers to deliver the miRNA mimics/inhibitors to the tumor, attempts have also been made in preclinical studies to screen for small molecule drugs that modulate miRNA expression and function by luciferase-based reporter assay[179]. These compounds do not inhibit target mRNA recognition by the miRNAs, but they instead modulate the expression of the targeted miRNAs. With potential application in treating CRC, diazobenzene and its derivatives were identified to specifically inhibit miR-21 expression[179].

Most recently, aberrant expression of the lipid metabolic enzymes, acyl-CoA synthetase/stearoyl-CoA desaturase (ACSL/SCD) have been shown in CRC patients to promote cancer migration and invasion[180]. miR-19b-1 was found to a major regulator of ACSL/SCD expression. Importantly, lower miR-19b-1 expression and higher ACSL/SCD levels in tumor specimens from CRC patients were strongly associated with shorter disease-free survival[180]. Therefore, miRNA replacement therapy was proposed to increase the expression level of miR-19b-1 in colorectal tissue to improve the prognosis. Interestingly, since induction of miR-19b-1 expression has been achieved by a low protein diet in piglets[181], dietary modulation of this particular miRNA may also be feasible. More *in vivo* investigation is needed to test the efficacy and safety before we might benefit from miR-19b-1 replacement therapy in the management of CRC.

HURDLES IN MODULATING MIRNAS TO TREAT CRC

Since miRNAs only require partial sequence complementarity to target mRNA, non-specific targeting of bystander mRNAs may lead to off-target effects, toxicity and/or unfavorable immune response. Moreover, rapid degradation of miRNAs or anti-miRNAs by cellular nucleases and poor cellular uptake are also preventing the development of successful miRNA-based gene therapies. Furthermore, the administration of anti-miRs *in vivo* with an aim to block oncogenic miRNAs may adversely affect physiological functions normally regulated by these miRNAs. Thus, specific site or cell targeting will be needed to avoid the potential adverse effects. The lowest optimum concentration of miRNAs and appropriate delivery systems, such as viral and/or non-viral vectors, are critical factors required to achieve the desired therapeutic goal while minimizing adverse effects.

CONCLUSION

Accumulating evidence suggests that miRNAs play a crucial role in the regulation of genes driving CRC initiation, progression and metastasis. Differential expression of miRNAs in CRC has provided opportunities for their applications in diagnosis, prognostic prediction and stratification of risk groups. While numerous miRNA biomarkers have been reported to predict prognosis and drug response in CRC patients, it is interesting to note that the miRNA signature identified in different studies usually do not overlap and, in certain cases, they were even found to be contradictory[182]. The different selection criteria for patients, collection method and processing of biological samples, and detection methods may contribute to the different miRNA signatures obtained. To obtain reliable results, precaution should be taken to prevent the degradation of miRNAs during sample

processing. It may be helpful to use these novel miRNA signatures together with the conventional biomarkers, such as CEA, to increase the sensitivity and specificity. As novel therapeutic targets, underexpressed miRNAs can be restored, whereas overexpressed miRNAs can be inhibited. Different delivery systems of these miRNA modulating moieties have been extensively studied in cell culture and animal models. Despite adverse effects having yielded a discouraging initial clinical experience with miR-34 replacement therapy, the correction of miRNA dysregulation is a promising therapeutic approach for the treatment of CRC; still, it is necessary to resolve the bystander effects and address toxicity concerns prior to large-scale clinical use.

REFERENCES

1   **Arnold M**, Sierra MS, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global patterns and trends in colorectal cancer incidence and mortality. *Gut* 2017; **66**: 683-691 [PMID: 26818619]

2   **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* 2018; **68**: 7-30 [PMID: 29313949 DOI: 10.3322/caac.21442]

3   **Fearon ER**, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61**: 759-767 [PMID: 2188735]

4   **DA Silva FC**, Wernhoff P, Dominguez-Barrera C, Dominguez-Valentin M. Update on Hereditary Colorectal Cancer. *Anticancer Res* 2016; **36**: 4399-4405 [PMID: 27630275 DOI: 10.21873/anticanres.10983]

5   **Peters U**, Bien S, Zubair N. Genetic architecture of colorectal cancer. *Gut* 2015; **64**: 1623-1636 [PMID: 26187503 DOI: 10.1136/gutjnl-2013-306705]

6   **Graham JS**, Cassidy J. Adjuvant therapy in colon cancer. *Expert Rev Anticancer Ther* 2012; **12**: 99-109 [PMID: 22149436]

7   **Yalcin S**, Trad D, Kader YA, Halawani H, Demir OG, Mall R, Meshcheryakov A, Nasr F, Nosworthy A, Osinsky D, Tumanova A, Turhal S, Tejpar S, Köhne CH. Personalized treatment is better than one treatment fits all in the management of patients with mCRC: a consensus statement. *Future Oncol* 2014; **10**: 2643-2657 [PMID: 25531050 DOI: 10.2217/fon.14.203]

8   **Michael MZ**, O' Connor SM, van Holst Pellekaan NG, Young GP, James RJ. Reduced accumulation of specific microRNAs in colorectal neoplasia. *Mol Cancer Res* 2003; **1**: 882-891 [PMID: 14573789]

9   **Bartel DP**. MicroRNAs: target recognition and regulatory functions. *Cell* 2009; **136**: 215-233 [PMID: 19167326 DOI: 10.1016/j.cell.2009.01.002]

10 **Lewis BP**, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005; **120**: 15-20 [PMID: 15652477 DOI: 10.1016/j.cell.2004.12.035]

11 **Segditsas S**, Tomlinson I. Colorectal cancer and genetic alterations in the Wnt pathway. *Oncogene* 2006; **25**: 7531-7537 [PMID: 17143297]

12 **Li T**, Lai Q, Wang S, Cai J, Xiao Z, Deng D, He L, Jiao H, Ye Y, Liang L, Ding Y, Liao W. MicroRNA-224 sustains Wnt/β-catenin signaling and promotes aggressive phenotype of colorectal cancer. *J Exp Clin Cancer Res* 2016; **35**: 21 [PMID: 26822534 DOI: 10.1186/s13046-016-0287-1]

13 **Medema JP**. Targeting the Colorectal Cancer Stem Cell. *N Engl J Med* 2017; **377**: 888-890 [PMID: 28854096]

14 **Vermeulen L**, De Sousa E Melo F, van der Heijden M, Cameron K, de Jong JH, Borovski T, Tuynman JB, Todaro M, Merz C, Rodermond H, Sprick MR, Kemper K, Richel DJ, Stassi G, Medema JP. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nat Cell Biol* 2010; **12**: 468-476 [PMID: 20418870 DOI: 10.1038/ncb2048]

15 **Le Grand F**, Jones AE, Seale V, Scimè A, Rudnicki MA. Wnt7a activates the planar cell polarity pathway to drive the symmetric expansion of satellite stem cells. *Cell Stem Cell* 2009; **4**: 535-547 [PMID: 19497282 DOI: 10.1016/j]

16 **Hwang WL**, Jiang JK, Yang SH, Huang TS, Lan HY, Teng HW, Yang CY, Tsai YP, Lin CH, Wang HW, Yang MH. MicroRNA-146a directs the symmetric division of Snail-dominant colorectal cancer stem cells. *Nat Cell Biol* 2014; **16**: 268-280 [PMID: 24561623 DOI: 10.1038/ncb2910]

17 **Velho S**, Oliveira C, Ferreira A, Ferreira AC, Suriano G, Schwartz S Jr, Duval A, Carneiro F, Machado JC, Hamelin R, Seruca R. The prevalence of PIK3CA mutations in gastric and colon cancer. *Eur J Cancer* 2005; **41**: 1649-1654 [PMID: 15994075 DOI: 10.1016/j.ejca.2005.04.022]

18 **Arcaroli JJ**, Quackenbush KS, Powell RW, Pitts TM, Spreafico A, Varella-Garcia M, Bemis L, Tan AC, Reinemann JM, Touban BM, Dasari A, Eckhardt SG, Messersmith WA. Common PIK3CA mutants and a novel 3' UTR mutation are associated with increased sensitivity to saracatinib. *Clin Cancer Res* 2012; **18**: 2704-2714 [PMID: 22553375]

19 **Guo C**, Sah JF, Beard L, Willson JK, Markowitz SD, Guda K. The noncoding RNA, miR-126, suppresses the growth of neoplastic cells by targeting phosphatidylinositol 3-kinase signaling and is frequently lost in colon cancers. *Genes Chromosomes Cancer* 2008; **47**: 939-946 [PMID: 18663744 DOI: 10.1002/gcc.20596]

20 **Cekaite L**, Eide PW, Lind GE, Skotheim RI, Lothe RA. MicroRNAs as growth regulators, their function and biomarker status in colorectal cancer. *Oncotarget* 2016; **7**: 6476-6505 [PMID: 26623728]

21 **Lièvre A**, Bachet JB, Boige V, Cayre A, Le Corre D, Buc E, Ychou M, Bouché O, Landi B, Louvet C, André T, Bibeau F, Diebold MD, Rougier P, Ducreux M, Tomasic G, Emile JF, Penault-Llorca F, Laurent-Puig P. KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. *J Clin Oncol* 2008; **26**: 374-379 [PMID: 18202412]

22 **Sebio A**, Paré L, Páez D, Salazar J, González A, Sala N, del Río E, Martín-Richard M, Tobeña M, Barnadas A, Baiget M. The LCS6 polymorphism in the binding site of let-7 microRNA to the KRAS 3'-untranslated region: its role in the efficacy of anti-EGFR-based therapy in metastatic colorectal cancer patients. *Pharmacogenet Genomics* 2013; **23**: 142-147 [PMID: 23324806 DOI: 10.1097/FPC.0b013e32835d9b0b]

23 **Tsang WP**, Kwok TT. The miR-18a* microRNA functions as a potential tumor suppressor by targeting on K-Ras. *Carcinogenesis* 2009; **30**: 953-959 [PMID: 19372139 DOI: 10.1093/carcin/bgp094]

24 **Liao WT**, Ye YP, Zhang NJ, Li TT, Wang SY, Cui YM, Qi L, Wu P, Jiao HL, Xie YJ, Zhang C, Wang JX, Ding YQ. MicroRNA-30b functions as a tumour suppressor in human colorectal cancer by targeting KRAS, PIK3CD and BCL2. *J Pathol* 2014; **232**: 415-427 [PMID: 24293274 DOI: 10.1002/path.4309]

25 **Chen X**, Guo X, Zhang H, Xiang Y, Chen J, Yin Y, Cai X, Wang K, Wang G, Ba Y, Zhu L, Wang J, Yang R, Zhang Y, Ren Z, Zen K, Zhang J, Zhang CY. Role of miR-143 targeting KRAS in colorectal tumorigenesis. *Oncogene* 2009; **28**: 1385-1392 [PMID: 19137007 DOI: 10.1038/omc/2008.474]

26 **Pagliuca A**, Valvo C, Fabrizi E, di Martino S, Biffoni M, Runci D, Forte S, De Maria R, Ricci-Vitiani L. Analysis of the combined action of miR-143 and miR-145 on oncogenic pathways in colorectal cancer cells reveals a coordinate program of gene repression. *Oncogene* 2013; **32**: 4806-4813 [PMID: 23128394 DOI: 10.1038/onc.2012.495]

27 **Grady WM**, Myeroff LL, Swinler SE, Rajput A, Thiagalingam S, Lutterbaugh JD, Neumann A, Brattain MG, Chang J, Kim SJ, Kinzler KW, Vogelstein B, Willson JK, Markowitz S. Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. *Cancer Res* 1999; **59**: 320-324 [PMID: 9927040]

28   **Grady WM**, Markowitz SD. Genetic and epigenetic alterations in colon cancer. *Annu Rev Genomics Hum Genet* 2002; **3**: 101-128 [PMID: 12142355]

29   **Yu Y**, Kanwar SS, Patel BB, Oh PS, Nautiyal J, Sarkar FH, Majumdar AP. MicroRNA-21 induces stemness by downregulating transforming growth factor beta receptor 2 (TGFβR2) in colon cancer cells. *Carcinogenesis* 2012; **33**: 68-76 [PMID: 22072622 DOI: 10.1093/carcin/bgr246]

30   **Feng B**, Dong TT, Wang LL, Zhou HM, Zhao HC, Dong F, Zheng MH. Colorectal cancer migration and invasion initiated by microRNA-106a. *PLoS One* 2012; **7**: e43452 [PMID: 22912877 DOI: 10.1371/journal.pone.0043452]

31   **Zhang W**, Zhang T, Jin R, Zhao H, Hu J, Feng B, Zang L, Zheng M, Wang M. MicroRNA-301a promotes migration and invasion by targeting TGFBR2 in human colorectal cancer. *J Exp Clin Cancer Res* 2014; **33**: 113 [PMID: 25551793 DOI: 10.1186/s13046-014-0113-6]

32   **Li Q**, Zou C, Zou C, Han Z, Xiao H, Wei H, Wang W, Zhang L, Zhang X, Tang Q, Zhang C, Tao J, Wang X, Gao X. MicroRNA-25 functions as a potential tumor suppressor in colon cancer by targeting Smad7. *Cancer Lett* 2013; **335**: 168-174 [PMID: 23435373 DOI: 10.1016/j.canlet.2013.02.029]

33   **Zhang F**, Luo Y, Shao Z, Xu L, Liu X, Niu Y, Shi J, Sun X, Liu Y, Ding Y, Zhao L. MicroRNA-187, a downstream effector of TGFβ pathway, suppresses Smad-mediated epithelial-mesenchymal transition in colorectal cancer. *Cancer Lett* 2016; **373**: 203-213 [PMID: 26820227 DOI: 10.1016/j.canlet.2016.01.037]

34   **Singh A**, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene* 2010; **29**: 4741-4751 [PMID: 20531305 DOI: 10.1038/onc.2010.215]

35   **Carstens JL**, Lovisa S, Kalluri R. Microenvironment-dependent cues trigger miRNA-regulated feedback loop to facilitate the EMT/MET switch. *J Clin Invest* 2014; **124**: 1458-1460 [PMID: 24642461]

36   **Zhang JX**, Mai SJ, Huang XX, Wang FW, Liao YJ, Lin MC, Kung HF, Zeng YX, Xie D. MiR-29c mediates epithelial-to-mesenchymal transition in human colorectal carcinoma metastasis via PTP4A and GNA13 regulation of β-catenin signaling. *Ann Oncol* 2014; **25**: 2196-2204 [PMID: 25193986 DOI: 10.1093/annonc/mdu439]

37   **Hur K**, Toiyama Y, Takahashi M, Balaguer F, Nagasaka T, Koike J, Hemmi H, Koi M, Boland CR, Goel A. MicroRNA-200c modulates epithelial-to-mesenchymal transition (EMT) in human colorectal cancer metastasis. *Gut* 2013; **62**: 1315-1326 [PMID: 22735571 DOI: 10.1136/gutjnl-2011-301846]

38   **Guo YH**, Wang LQ, Li B, Xu H, Yang JH, Zheng LS, Yu P, Zhou AD, Zhang Y, Xie SJ, Liang ZR, Zhang CM, Zhou H, Qu LH. Wnt/β-catenin pathway transactivates microRNA-150 that promotes EMT of colorectal cancer cells by suppressing CREB signaling. *Oncotarget* 2016; **7**: 42513-42526 [PMID: 27285761 DOI: 10.18632/oncotarget.9893]

39   **Zauber AG**, Winawer SJ, O'Brien MJ, Lansdorp-Vogelaar I, van Ballegooijen M, Hankey BF, Shi W, Bond JH, Schapiro M, Panish JF, Stewart ET, Waye JD. Colonoscopic polypectomy and long-term prevention of colorectal-cancer deaths. *N Engl J Med* 2012; **366**: 687-696 [PMID: 22356322 DOI: 10.1056/NEJMoa1100370]

40   **Nishihara R**, Wu K, Lochhead P, Morikawa T, Liao X, Qian ZR, Inamura K, Kim SA, Kuchiba A, Yamauchi M, Imamura Y, Willett WC, Rosner BA, Fuchs CS, Giovannucci E, Ogino S, Chan AT. Long-term colorectal-cancer incidence and mortality after lower endoscopy. *N Engl J Med* 2013; **369**: 1095-1105 [PMID: 24047059]

41   **Akao Y**, Nakagawa Y, Naoe T. MicroRNAs 143 and 145 are possible common onco-microRNAs in human cancers. *Oncol Rep* 2006; **16**: 845-850 [PMID: 16969504]

42   **Uratani R**, Toiyama Y, Kitajima T, Kawamura M, Hiro J, Kobayashi M, Tanaka K, Inoue Y, Mohri Y, Mori T, Kato T, Goel A, Kusunoki M. Diagnostic Potential of Cell-Free and Exosomal MicroRNAs in the Identification of Patients with High-Risk

43   **Moridikia A**, Mirzaei H, Sahebkar A, Salimian J. MicroRNAs: Potential candidates for diagnosis and treatment of colorectal cancer. *J Cell Physiol* 2018; **233**: 901-913 [PMID: 28092102]

44   **Carter JV**, Galbraith NJ, Yang D, Burton JF, Walker SP, Galandiuk S. Blood-based microRNAs as biomarkers for the diagnosis of colorectal cancer: a systematic review and meta-analysis. *Br J Cancer* 2017; **116**: 762-774 [PMID: 28152545 DOI: 10.1038/bjc.2017.12]

45   **Pan C**, Yan X, Li H, Huang L, Yin M, Yang Y, Gao R, Hong L, Ma Y, Shi C, Qin H, Zhang P. Systematic literature review and clinical validation of circulating microRNAs as diagnostic biomarkers for colorectal cancer. *Oncotarget* 2017; **8**: 68317-68328 [PMID: 28978119 DOI: 10.18632/oncotarget.19344]

46   **Vatandoost N**, Ghanbari J, Mojaver M, Avan A, Ghayour-Mobarhan M, Nedaeinia R, Salehi R. Early detection of colorectal cancer: from conventional methods to novel biomarkers. *J Cancer Res Clin Oncol* 2016; **142**: 341-351 [PMID: 25687380 DOI: 10.1007/s00432-015-1928-z]

47   **Clancy C**, Joyce MR, Kerin MJ. The use of circulating microRNAs as diagnostic biomarkers in colorectal cancer. *Cancer Biomark* 2015; **15**: 103-113 [PMID: 25547322]

48   **Sood P**, Krek A, Zavolan M, Macino G, Rajewsky N. Cell-type-specific signatures of microRNAs on target mRNA expression. *Proc Natl Acad Sci USA* 2006; **103**: 2746-2751 [PMID: 16477010 DOI: 10.1073/pnas.0511045103]

49   **Ludwig N**, Leidinger P, Becker K, Backes C, Fehlmann T, Pallasch C, Rheinheimer S, Meder B, Stähler C, Meese E, Keller A. Distribution of miRNA expression across human tissues. *Nucleic Acids Res* 2016; **44**: 3865-3877 [PMID: 26921406 DOI: 10.1093/nar/gkw116]

50   **Sha D**, Lee AM, Shi Q, Alberts SR, Sargent DJ, Sinicrope FA, Diasio RB. Association study of the let-7 miRNA-complementary site variant in the 3' untranslated region of the KRAS gene in stage III colon cancer (NCCTG N0147 Clinical Trial). *Clin Cancer Res* 2014; **20**: 3319-3327 [PMID: 24727325 DOI: 10.1158/1078-0432.CCR-14-0069]

51   **Yu G**, Tang JQ, Tian ML, Li H, Wang X, Wu T, Zhu J, Huang SJ, Wan YL. Prognostic values of the miR-17-92 cluster and its paralogs in colon cancer. *J Surg Oncol* 2012; **106**: 232-237 [PMID: 22065543 DOI: 10.1002/jso.22138]

52   **Tsuchida A**, Ohno S, Wu W, Borjigin N, Fujita K, Aoki T, Ueda S, Takanashi M, Kuroda M. miR-92 is a key oncogenic component of the miR-17-92 cluster in colon cancer. *Cancer Sci* 2011; **102**: 2264-2271 [PMID: 21883694 DOI: 10.1111/j.1349-7006.2011.02081.x]

53   **Peric D**, Chvalova K, Rousselet G. Identification of microprocessor-dependent cancer cells allows screening for growth-sustaining micro-RNAs. *Oncogene* 2012; **31**: 2039-2048 [PMID: 21909134 DOI: 10.1038/onc.2011.391]

54   **Liang Z**, Li Y, Huang K, Wagar N, Shim H. Regulation of miR-19 to breast cancer chemoresistance through targeting PTEN. *Pharm Res* 2011; **28**: 3091-3100 [PMID: 21853360 DOI: 10.1007/s11095-011-0570-y]

55   **Olive V**, Bennett MJ, Walker JC, Ma C, Jiang I, Cordon-Cardo C, Li QJ, Lowe SW, Hannon GJ, He L. miR-19 is a key oncogenic component of mir-17-92. *Genes Dev* 2009; **23**: 2839-2849 [PMID: 20008935 DOI: 10.1101/gad.1861409]

56   **Tili E**, Michaille JJ, Liu CG, Alder H, Taccioli C, Volinia S, Calin GA, Croce CM. GAM/ZFp/ZNF512B is central to a gene sensor circuitry involving cell-cycle regulators, TGF{beta} effectors, Drosha and microRNAs with opposite oncogenic potentials. *Nucleic Acids Res* 2010; **38**: 7673-7688 [PMID: 20639536 DOI: 10.1093/nar/gkg637]

57   **Ellermeier C**, Vang S, Cleveland K, Durand W, Resnick MB, Brodsky AS. Prognostic microRNA expression signature from examination of colorectal primary and metastatic tumors. *Anticancer Res* 2014; **34**: 3957-3967 [PMID: 25075017]

Colorectal Adenomas. *PLoS One* 2016; **11**: e0160722 [PMID: 27760147 DOI: 10.1371/journal.pone.0160722]

58   **Slaby O**, Svoboda M, Fabian P, Smerdova T, Knoflickova D, Bednarikova M, Nenutil R, Vyzula R. Altered expression of miR-21, miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer. *Oncology* 2007; **72**: 397-402 [PMID: 18196926 DOI: 10.1159/000113489]

59   **Shibuya H**, Iinuma H, Shimada R, Horiuchi A, Watanabe T. Clinicopathological and prognostic value of microRNA-21 and microRNA-155 in colorectal cancer. *Oncology* 2010; **79**: 313-320 [PMID: 21412018 DOI: 10.1159/000323283]

60   **Drusco A**, Nuovo GJ, Zanesi N, Di Leva G, Pichiorri F, Volinia S, Fernandez C, Antenucci A, Costinean S, Bottoni A, Rosito IA, Liu CG, Burch A, Acunzo M, Pekarsky Y, Alder H, Ciardi A, Croce CM. MicroRNA profiles discriminate among colon cancer metastasis. *PLoS One* 2014; **9**: e96670 [PMID: 24921248 DOI: 10.1371/journal.pone.0096670]

61   **Weissmann-Brenner A**, Kushnir M, Lithwick Yanai G, Aharonov R, Gibori H, Purim O, Kundel Y, Morgenstern S, Halperin M, Niv Y, Brenner B. Tumor microRNA-29a expression and the risk of recurrence in stage II colon cancer. *Int J Oncol* 2012; **40**: 2097-2103 [PMID: 22426940 DOI: 10.3892/ijo.2012.1403]

62   **Tang W**, Zhu Y, Gao J, Fu J, Liu C, Liu Y, Song C, Zhu S, Leng Y, Wang G, Chen W, Du P, Huang S, Zhou X, Kang J, Cui L. MicroRNA-29a promotes colorectal cancer metastasis by regulating matrix metalloproteinase 2 and E-cadherin via KLF4. *Br J Cancer* 2014; **110**: 450-458 [PMID: 24281002 DOI: 10.1038/bjc.2013.724]

63   **Locker GY**, Hamilton S, Harris J, Jessup JM, Kemeny N, Macdonald JS, Somerfield MR, Hayes DF, Bast RC Jr; ASCO. ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. *J Clin Oncol* 2006; **24**: 5313-5327 [PMID: 17060676 DOI: 10.1200/JCO.2006.08.2644]

64   **Mitchell PS**, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA* 2008; **105**: 10513-10518 [PMID: 18663219 DOI: 10.1073/pnas.0804549105]

65   **Turchinovich A**, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microRNA. *Nucleic Acids Res* 2011; **39**: 7223-7233 [PMID: 21609964 DOI: 10.1093/nar/gkr254]

66   **Valadi H**, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007; **9**: 654-659 [PMID: 17486113 DOI: 10.1038/ncb1596]

67   **Wang J**, Huang SK, Zhao M, Yang M, Zhong JL, Gu YY, Peng H, Che YQ, Huang CZ. Identification of a circulating microRNA signature for colorectal cancer detection. *PLoS One* 2014; **9**: e87451 [PMID: 24709885 DOI: 10.1371/journal.pone.0087451]

68   **Toiyama Y**, Takahashi M, Hur K, Nagasaka T, Tanaka K, Inoue Y, Kusunoki M, Boland CR, Goel A. Serum miR-21 as a diagnostic and prognostic biomarker in colorectal cancer. *J Natl Cancer Inst* 2013; **105**: 849-859 [PMID: 23704278 DOI: 10.1093/jnci/djt101]

69   **Hollis M**, Nair K, Vyas A, Chaturvedi LS, Gambhir S, Vyas D. MicroRNAs potential utility in colon cancer: Early detection, prognosis, and chemosensitivity. *World J Gastroenterol* 2015; **21**: 8284-8292 [PMID: 26217080 DOI: 10.3748/wjg.v21.i27.8284]

70   **Cheng H**, Zhang L, Cogdell DE, Zheng H, Schetter AJ, Nykter M, Harris CC, Chen K, Hamilton SR, Zhang W. Circulating plasma MiR-141 is a novel biomarker for metastatic colon cancer and predicts poor prognosis. *PLoS One* 2011; **6**: e17745 [PMID: 21445232 DOI: 10.1371/journal.pone.0017745]

71   **Fang Z**, Tang J, Bai Y, Lin H, You H, Jin H, Lin L, You P, Li J, Dai Z, Liang X, Su Y, Hu Q, Wang F, Zhang ZY. Plasma levels of microRNA-24, microRNA-320a, and microRNA-423-5p are potential biomarkers for colorectal carcinoma. *J Exp Clin Cancer Res* 2015; **34**: 86 [PMID: 26297223 DOI: 10.1186/s13046-015-0198-6]

72   **Ahmed FE**, Jeffries CD, Vos PW, Flake G, Nuovo GJ, Sinar DR, Naziri W, Marcuard SP. Diagnostic microRNA markers for screening sporadic human colon cancer and active ulcerative colitis in stool and tissue. *Cancer Genomics Proteomics* 2009; **6**: 281-295 [PMID: 19996134]

73   **Ahlquist DA**. Molecular detection of colorectal neoplasia. *Gastroenterology* 2010; **138**: 2127-2139 [PMID: 20420950 DOI: 10.1053/j.gastro.2010.01.055]

74   **Ahmed FE**, Ahmed NC, Vos PW, Bonnerup C, Atkins JN, Casey M, Nuovo GJ, Naziri W, Wiley JE, Mota H, Allison RR. Diagnostic microRNA markers to screen for sporadic human colon cancer in stool: I. Proof of principle. *Cancer Genomics Proteomics* 2013; **10**: 93-113 [PMID: 23741026]

75   **Wu CW**, Ng SC, Dong Y, Tian L, Ng SS, Leung WW, Law WT, Yau TO, Chan FK, Sung JJ, Yu J. Identification of microRNA-135b in stool as a potential noninvasive biomarker for colorectal cancer and adenoma. *Clin Cancer Res* 2014; **20**: 2994-3002 [PMID: 24691020 DOI: 10.1158/1078-0432.CCR-13-1750]

76   **Braicu C**, Tomuleasa C, Monroig P, Cucuianu A, Berindan-Neagoe I, Calin GA. Exosomes as divine messengers: are they the Hermes of modern molecular oncology? *Cell Death Differ* 2015; **22**: 34-45 [PMID: 25236394 DOI: 10.1038/cdd.2014.130]

77   **Ji H**, Greening DW, Barnes TW, Lim JW, Tauro BJ, Rai A, Xu R, Adda C, Mathivanan S, Zhao W, Xue Y, Xu T, Zhu HJ, Simpson RJ. Proteome profiling of exosomes derived from human primary and metastatic colorectal cancer cells reveal differential expression of key metastatic factors and signal transduction components. *Proteomics* 2013; **13**: 1672-1686 [PMID: 23585443 DOI: 10.1002/pmic.201200562]

78   **Matsumura T**, Sugimachi K, Iinuma H, Takahashi Y, Kurashige J, Sawada G, Ueda M, Uchi R, Ueo H, Takano Y, Shinden Y, Eguchi H, Yamamoto H, Doki Y, Mori M, Ochiya T, Mimori K. Exosomal microRNA in serum is a novel biomarker of recurrence in human colorectal cancer. *Br J Cancer* 2015; **113**: 275-281 [PMID: 26057451 DOI: 10.1038/bjc.2015.201]

79   **Monzo M**, Santasusagna S, Moreno I, Martinez F, Hernández R, Muñoz C, Castellano JJ, Moreno J, Navarro A. Exosomal microRNAs isolated from plasma of mesenteric veins linked to liver metastases in resected patients with colon cancer. *Oncotarget* 2017; **8**: 30859-30869 [PMID: 28415718 DOI: 10.18632/oncotarget.16103]

80   NIH consensus conference. Adjuvant therapy for patients with colon and rectal cancer. *JAMA* 1990; **264**: 1444-1450 [PMID: 2202842]

81   **Quasar Collaborative Group.**, Gray R, Barnwell J, McConkey C, Hills RK, Williams NS, Kerr DJ. Adjuvant chemotherapy versus observation in patients with colorectal cancer: a randomised study. *Lancet* 2007; **370**: 2020-2029 [PMID: 18083404 DOI: 10.1016/S0140-6736(07)61866-2]

82   **Labianca R**, Nordlinger B, Beretta GD, Brouquet A, Cervantes A; ESMO Guidelines Working Group. Primary colon cancer: ESMO Clinical Practice Guidelines for diagnosis, adjuvant treatment and follow-up. *Ann Oncol* 2010; **21** Suppl 5: v70-v77 [PMID: 20555107 DOI: 10.1093/annonc/mdg168]

83   **Agesen TH**, Sveen A, Merok MA, Lind GE, Nesbakken A, Skotheim RI, Lothe RA. ColoGuideEx: a robust gene classifier specific for stage II colorectal cancer prognosis. *Gut* 2012; **61**: 1560-1567 [PMID: 22213796 DOI: 10.1136/gutjnl-2011-301179]

84   **Schetter AJ**, Nguyen GH, Bowman ED, Mathé EA, Yuen ST, Hawkes JE, Croce CM, Leung SY, Harris CC. Association of inflammation-related and microRNA gene expression with cancer-specific mortality of colon adenocarcinoma. *Clin Cancer Res* 2009; **15**: 5878-5887 [PMID: 19737943 DOI: 10.1158/1078-0432.CCR-09-0627]

85   **Kjaer-Frifeldt S**, Hansen TF, Nielsen BS, Joergensen S, Lindebjerg J, Soerensen FB, dePont Christensen R, Jakobsen A; Danish Colorectal Cancer Group. The prognostic importance of

miR-21 in stage II colon cancer: a population-based study. *Br J Cancer* 2012; **107**: 1169-1174 [PMID: 23011541 DOI: 10.1038/bjc.2012.365]

86   **Zhang JX**, Song W, Chen ZH, Wei JH, Liao YJ, Lei J, Hu M, Chen GZ, Liao B, Lu J, Zhao HW, Chen W, He YL, Wang HY, Xie D, Luo JH. Prognostic and predictive value of a microRNA signature in stage II colon cancer: a microRNA expression analysis. *Lancet Oncol* 2013; **14**: 1295-1306 [PMID: 24239208 DOI: 10.1016/S1470-2045(13)70491-1]

87   **Yamazaki N**, Koga Y, Taniguchi H, Kojima M, Kanemitsu Y, Saito N, Matsumura Y. High expression of miR-181c as a predictive marker of recurrence in stage II colorectal cancer. *Oncotarget* 2017; **8**: 6970-6983 [PMID: 28036302 DOI: 10.18632/oncotarget.14344]

88   **Salendo J**, Spitzner M, Kramer F, Zhang X, Jo P, Wolff HA, Kitz J, Kaulfuß S, Beißbarth T, Dobbelstein M, Ghadimi M, Grade M, Gaedcke J. Identification of a microRNA expression signature for chemoradiosensitivity of colorectal cancer cells, involving miRNAs-320a, -224, -132 and let7g. *Radiother Oncol* 2013; **108**: 451-457 [PMID: 23932154 DOI: 10.1016/j.radonc.2013.06.032]

89   **Caramés C**, Cristóbal I, Moreno V, del Puerto L, Moreno I, Rodriguez M, Marín JP, Correa AV, Hernández R, Zenzola V, Hernández T, León A, Martín JI, Sánchez-Fayos P, García-Olmo D, Rojo F, Goel A, Fernandez-Aceñero MJ, García-Foncillas J. MicroRNA-21 predicts response to preoperative chemoradiotherapy in locally advanced rectal cancer. *Int J Colorectal Dis* 2015; **30**: 899-906 [PMID: 25953218 DOI: 10.1007/s00384-015-2231-9]

90   **Schetter AJ**, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, Yanaihara N, Yuen ST, Chan TL, Kwong DL, Au GK, Liu CG, Calin GA, Croce CM, Harris CC. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA* 2008; **299**: 425-436 [PMID: 18230780 DOI: 10.1001/jama.299.4.425]

91   **Ma Y**, Zhang P, Wang F, Zhang H, Yang J, Peng J, Liu W, Qin H. miR-150 as a potential biomarker associated with prognosis and therapeutic outcome in colorectal cancer. *Gut* 2012; **61**: 1447-1453 [PMID: 22052060 DOI: 10.1136/qitjnl-2011-301122]

92   **Takahashi M**, Cuatrecasas M, Balaguer F, Hur K, Toiyama Y, Castells A, Boland CR, Goel A. The clinical significance of MiR-148a as a predictive biomarker in patients with advanced colorectal cancer. *PLoS One* 2012; **7**: e46684 [PMID: 23056401 DOI: 10.1371/journal.pone.0046684]

93   **Perez-Carbonell L**, Sinicrope FA, Alberts SR, Oberg AL, Balaguer F, Castells A, Boland CR, Goel A. MiR-320e is a novel prognostic biomarker in colorectal cancer. *Br J Cancer* 2015; **113**: 83-90 [PMID: 26035698 DOI: 10.1038/bjc.2015.168]

94   **Rasmussen MH**, Jensen NF, Tarpgaard LS, Qvortrup C, Rømer MU, Stenvang J, Hansen TP, Christensen LL, Lindebjerg J, Hansen F, Jensen BV, Hansen TF, Pfeiffer P, Brünner N, Ørntoft TF, Andersen CL. High expression of microRNA-625-3p is associated with poor response to first-line oxaliplatin based treatment of metastatic colorectal cancer. *Mol Oncol* 2013; **7**: 637-646 [PMID: 23506979 DOI: 10.1016/j.molonc.2013.02.016]

95   **Svoboda M**, Sana J, Fabian P, Kocakova I, Gombosova J, Nekvindova J, Radova L, Vyzula R, Slaby O. MicroRNA expression profile associated with response to neoadjuvant chemoradiotherapy in locally advanced rectal cancer patients. *Radiat Oncol* 2012; **7**: 195 [PMID: 23167930 DOI: 10.1186/1748-717X-7-195]

96   **Della Vittoria Scarpati G**, Falcetta F, Carlomagno C, Ubezio P, Marchini S, De Stefano A, Singh VK, D'Incalci M, De Placido S, Pepe S. A specific miRNA signature correlates with complete pathological response to neoadjuvant chemoradiotherapy in locally advanced rectal cancer. *Int J Radiat Oncol Biol Phys* 2012; **83**: 1113-1119 [PMID: 22172905 DOI: 10.1016/j.ijrobp.2011.09.030]

97   **Suto T**, Yokobori T, Yajima R, Morita H, Fujii T, Yamaguchi S, Altan B, Tsutsumi S, Asao T, Kuwano H. MicroRNA-7 expression in colorectal cancer is associated with poor prognosis and regulates cetuximab sensitivity via EGFR regulation. *Carcinogenesis* 2015; **36**: 338-345 [PMID: 25503932 DOI: 10.1093/carcin/bgu242]

98   **Mosakhani N**, Lahti L, Borze I, Karjalainen-Lindsberg ML, Sundström J, Ristamäki R, Osterlund P, Knuutila S, Sarhadi VK. MicroRNA profiling predicts survival in anti-EGFR treated chemorefractory metastatic colorectal cancer patients with wild-type KRAS and BRAF. *Cancer Genet* 2012; **205**: 545-551 [PMID: 23098991 DOI: 10.1016/j.cancergen.2012.08.003]

99   **Pichler M**, Winter E, Ress AL, Bauernhofer T, Gerger A, Kiesslich T, Lax S, Samonigg H, Hoefler G. miR-181a is associated with poor clinical outcome in patients with colorectal cancer treated with EGFR inhibitor. *J Clin Pathol* 2014; **67**: 198-203 [PMID: 24098024 DOI: 10.1136/jclinpath-2013-201904]

100   **Igarashi H**, Kurihara H, Mitsuhashi K, Ito M, Okuda H, Kanno S, Naito T, Yoshii S, Takahashi H, Kusumi T, Hasegawa T, Sukawa Y, Adachi Y, Okita K, Hirata K, Imamura Y, Baba Y, Imai K, Suzuki H, Yamamoto H, Nosho K, Shinomura Y. Association of MicroRNA-31-5p with Clinical Efficacy of Anti-EGFR Therapy in Patients with Metastatic Colorectal Cancer. *Ann Surg Oncol* 2015; **22**: 2640-2648 [PMID: 25472647 DOI: 10.1245/s10434-014-4264-7]

101   **Cappuzzo F**, Sacconi A, Landi L, Ludovini V, Biagioni F, D'Incecco A, Capodanno A, Salvini J, Corgna E, Cupini S, Barbara C, Fontanini G, Crinò L, Blandino G. MicroRNA signature in metastatic colorectal cancer patients treated with anti-EGFR monoclonal antibodies. *Clin Colorectal Cancer* 2014; **13**: 37-45.e4 [PMID: 24503111 DOI: 10.1016/j.clcc.2013.11.006]

102   **Ruzzo A**, Graziano F, Vincenzi B, Canestrari E, Perrone G, Galluccio N, Catalano V, Loupakis F, Rabitti C, Santini D, Tonini G, Fiorentini G, Rossi D, Falcone A, Magnani M. High let-7a microRNA levels in KRAS-mutated colorectal carcinomas may rescue anti-EGFR therapy effects in patients with chemotherapy-refractory metastatic disease. *Oncologist* 2012; **17**: 823-829 [PMID: 22584434]

103   **Mekenkamp LJ**, Tol J, Dijkstra JR, de Krijger I, Vink-Börger ME, van Vliet S, Teerenstra S, Kamping E, Verwiel E, Koopman M, Meijer GA, van Krieken JH, Kuiper R, Punt CJ, Nagtegaal ID. Beyond KRAS mutation status: influence of KRAS copy number status and microRNAs on clinical outcome to cetuximab in metastatic colorectal cancer patients. *BMC Cancer* 2012; **12**: 292 [PMID: 22804917 DOI: 10.1186/1471-2407-12-292]

104   **Hansen TF**, Carlsen AL, Heegaard NH, Sørensen FB, Jakobsen A. Changes in circulating microRNA-126 during treatment with chemotherapy and bevacizumab predicts treatment response in patients with metastatic colorectal cancer. *Br J Cancer* 2015; **112**: 624-629 [PMID: 25584492 DOI: 10.1038/bjc.2014.652]

105   **Kjersem JB**, Ikdahl T, Lingjaerde OC, Guren T, Tveit KM, Kure EH. Plasma microRNAs predicting clinical outcome in metastatic colorectal cancer patients receiving first-line oxaliplatin-based treatment. *Mol Oncol* 2014; **8**: 59-67 [PMID: 24119443 DOI: 10.1016/j.molonc.2013.09.001]

106   **Zhang J**, Zhang K, Bi M, Jiao X, Zhang D, Dong Q. Circulating microRNA expressions in colorectal cancer as predictors of response to chemotherapy. *Anticancer Drugs* 2014; **25**: 346-352 [PMID: 24304648 DOI: 10.1097/CAD.0000000000000049]

107   **Chen J**, Wang W, Zhang Y, Chen Y, Hu T. Predicting distant metastasis and chemoresistance using plasma miRNAs. *Med Oncol* 2014; **31**: 799 [PMID: 24310813 DOI: 10.1007/s12032-013-0799-x]

108   **Schou JV**, Rossi S, Jensen BV, Nielsen DL, Pfeiffer P, Høgdall E, Yilmaz M, Tejpar S, Delorenzi M, Kruhøffer M, Johansen JS. miR-345 in metastatic colorectal cancer: a non-invasive biomarker for clinical outcome in non-KRAS mutant patients treated with 3rd line cetuximab and irinotecan. *PLoS One* 2014; **9**: e99886 [PMID: 24940606 DOI: 10.1371/journal.pone.0099886]

109   **Landi D**, Gemignani F, Landi S. Role of variations within microRNA-binding sites in cancer. *Mutagenesis* 2012; **27**: 205-210 [PMID: 22294768]

110   **Ryan BM**, Robles AI, Harris CC. Genetic variation in microRNA

networks: the implications for cancer research. *Nat Rev Cancer* 2010; **10**: 389-402 [PMID: 20495573 DOI: 10.1038/nrc2867]

111 **Vodicka P**, Pardini B, Vymetalkova V, Naccarati A. "Polymorphisms in non-coding RNA genes and their targets sites as risk factors of sporadic colorectal cancer," in Non-Coding RNAs in Colorectal Cancer, vol.937 of Advances in Experimental Medicine and Biology, pp. 123-149, Springer International, Cham, Switzerland, 2016.

112 **Yang YP**, Ting WC, Chen LM, Lu TL, Bao BY. Polymorphisms in MicroRNA Binding Sites Predict Colorectal Cancer Survival. *Int J Med Sci* 2017; **14**: 53-57 [PMID: 28138309 DOI: 10.7150/ijms.17027]

113 **Bahrami-B F**, Ataie-Kachoie P, Pourgholami MH, Morris DL. p70 Ribosomal protein S6 kinase (Rps6kb1): an update. *J Clin Pathol* 2014; **67**: 1019-1025 [PMID: 25100792 DOI: 10.1136/jclinpath-2014-202560]

114 **Scanlan MJ**, Gordan JD, Williamson B, Stockert E, Bander NH, Jongeneel V, Gure AO, Jäger D, Jäger E, Knuth A, Chen YT, Old LJ. Antigens recognized by autologous antibody in patients with renal-cell carcinoma. *Int J Cancer* 1999; **83**: 456-464 [PMID: 10508479]

115 **Zhang W**, Winder T, Ning Y, Pohl A, Yang D, Kahn M, Lurje G, Labonte MJ, Wilson PM, Gordon MA, Hu-Lieskovan S, Mauro DJ, Langer C, Rowinsky EK, Lenz HJ. A let-7 microRNA-binding site polymorphism in 3'-untranslated region of KRAS gene predicts response in wild-type KRAS patients with metastatic colorectal cancer treated with cetuximab monotherapy. *Ann Oncol* 2011; **22**: 104-109 [PMID: 20603437 DOI: 10.1093/annonc/mdg315]

116 **Aslam MI**, Patel M, Singh B, Jameson JS, Pringle JH. MicroRNA manipulation in colorectal cancer cells: from laboratory to clinical application. *J Transl Med* 2012; **10**: 128 [PMID: 22716183 DOI: 10.1186/1479-5876-10-128]

117 **Shah MY**, Calin GA. MicroRNAs as therapeutic targets in human cancers. *Wiley Interdiscip Rev RNA* 2014; **5**: 537-548 [PMID: 24687772 DOI: 10.1002/wrna.1229]

118 **Lin PL**, Wu DW, Huang CC, He TY, Chou MC, Sheu GT, Lee H. MicroRNA-21 promotes tumour malignancy via increased nuclear translocation of β-catenin and predicts poor outcome in APC-mutated but not in APC-wild-type colorectal cancer. *Carcinogenesis* 2014; **35**: 2175-2182 [PMID: 24832083 DOI: 10.1093/carcin/bgu110]

119 **Asangani IA**, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, Allgayer H. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene* 2008; **27**: 2128-2136 [PMID: 17968323 DOI: 10.1038/sj.onc.1210856]

120 **Valeri N**, Gasparini P, Braconi C, Paone A, Lovat F, Fabbri M, Sumani KM, Alder H, Amadori D, Patel T, Nuovo GJ, Fishel R, Croce CM. MicroRNA-21 induces resistance to 5-fluorouracil by down-regulating human DNA MutS homolog 2 (hMSH2). *Proc Natl Acad Sci USA* 2010; **107**: 21098-21103 [PMID: 21078976 DOI: 10.1073/pnas.1015541107]

121 **Wagenaar TR**, Zabludoff S, Ahn SM, Allerson C, Arlt H, Baffa R, Cao H, Davis S, Garcia-Echeverria C, Gaur R, Huang SM, Jiang L, Kim D, Metz-Weidmann C, Pavlicek A, Pollard J, Reeves J, Rocnik JL, Scheidler S, Shi C, Sun F, Tolstykh T, Weber W, Winter C, Yu E, Yu Q, Zheng G, Wiederschain D. Anti-miR-21 Suppresses Hepatocellular Carcinoma Growth via Broad Transcriptional Network Deregulation. *Mol Cancer Res* 2015; **13**: 1009-1021 [PMID: 25758165 DOI: 10.1158/1541-7786.MCR-14-0703]

122 **Hermeking H**. MicroRNAs in the p53 network: micromanagement of tumour suppression. *Nat Rev Cancer* 2012; **12**: 613-626 [PMID: 22898542 DOI: 10.1038/nrc3318]

123 **Chang TC**, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH, Feldmann G, Yamakuchi M, Ferlito M, Lowenstein CJ, Arking DE, Beer MA, Maitra A, Mendell JT. Transactivation of miR-34a by p53 broadly influences gene expression and promotes

apoptosis. *Mol Cell* 2007; **26**: 745-752 [PMID: 17540599 DOI: 10.1016/j.molcel.2007.05.010]

124 **Nugent M**, Miller N, Kerin MJ. Circulating miR-34a levels are reduced in colorectal cancer. *J Surg Oncol* 2012; **106**: 947-952 [PMID: 22648208 DOI: 10.1002/jso.23174]

125 **Hahn S**, Jackstadt R, Siemens H, Hünten S, Hermeking H. SNAIL and miR-34a feed-forward regulation of ZNF281/ZBP99 promotes epithelial-mesenchymal transition. *EMBO J* 2013; **32**: 3079-3095 [PMID: 24185900 DOI: 10.1038/emboj.2013.236]

126 **Rokavec M**, Öner MG, Li H, Jackstadt R, Jiang L, Lodygin D, Kaller M, Horst D, Ziegler PK, Schwitalla S, Slotta-Huspenina J, Bader FG, Greten FR, Hermeking H. IL-6R/STAT3/miR-34a feedback loop promotes EMT-mediated colorectal cancer invasion and metastasis. *J Clin Invest* 2014; **124**: 1853-1867 [PMID: 24642471 DOI: 10.1172/JCI73531]

127 **Strillacci A**, Valerii MC, Sansone P, Caggiano C, Sgromo A, Vittori L, Fiorentino M, Poggioli G, Rizzello F, Campieri M, Spisni E. Loss of miR-101 expression promotes Wnt/β-catenin signalling pathway activation and malignancy in colon cancer cells. *J Pathol* 2013; **229**: 379-389 [PMID: 22930392 DOI: 10.1002/path.4097]

128 **Nagel R**, le Sage C, Diosdado B, van der Waal M, Oude Vrielink JA, Bolijn A, Meijer GA, Agami R. Regulation of the adenomatous polyposis coli gene by the miR-135 family in colorectal cancer. *Cancer Res* 2008; **68**: 5795-5802 [PMID: 18632633 DOI: 10.1158/0008-5472.CAN-08-0951]

129 **Valeri N**, Braconi C, Gasparini P, Murgia C, Lampis A, Paulus-Hock V, Hart JR, Ueno L, Grivennikov SI, Lovat F, Paone A, Cascione L, Sumani KM, Veronese A, Fabbri M, Carasi S, Alder H, Lanza G, Gafa' R, Moyer MP, Ridgway RA, Cordero J, Nuovo GJ, Frankel WL, Rugge M, Fassan M, Groden J, Vogt PK, Karin M, Sansom OJ, Croce CM. MicroRNA-135b promotes cancer progression by acting as a downstream effector of oncogenic pathways in colon cancer. *Cancer Cell* 2014; **25**: 469-483 [PMID: 24735923 DOI: 10.1016/j.ccr.2014.03.006]

130 **Drebber U**, Lay M, Wedemeyer I, Vallböhmer D, Bollschweiler E, Brabender J, Mönig SP, Hölscher AH, Dienes HP, Odenthal M. Altered levels of the onco-microRNA 21 and the tumor-supressor microRNAs 143 and 145 in advanced rectal cancer indicate successful neoadjuvant chemoradiotherapy. *Int J Oncol* 2011; **39**: 409-415 [PMID: 21567082 DOI: 10.3892/ijo.2011.1036]

131 **Schetter AJ**, Okayama H, Harris CC. The role of microRNAs in colorectal cancer. *Cancer J* 2012; **18**: 244-252 [PMID: 22647361 DOI: 10.1097/PPO.0b013e318258b78f]

132 **Akao Y**, Nakagawa Y, Hirata I, Iio A, Itoh T, Kojima K, Nakashima R, Kitade Y, Naoe T. Role of anti-oncomirs miR-143 and -145 in human colorectal tumors. *Cancer Gene Ther* 2010; **17**: 398-408 [PMID: 20094072 DOI: 10.1038/cgt.2009.88]

133 **Huang L**, Fu L. Mechanisms of resistance to EGFR tyrosine kinase inhibitors. *Acta Pharm Sin B* 2015; **5**: 390-401 [PMID: 26579470 DOI: 10.1016/j.apsb.2015.07.001]

134 **Johnson SM**, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack FJ. RAS is regulated by the let-7 microRNA family. *Cell* 2005; **120**: 635-647 [PMID: 15766527 DOI: 10.1016/j.cell.2005.01.014]

135 **Hiraki M**, Nishimura J, Takahashi H, Wu X, Takahashi Y, Miyo M, Nishida N, Uemura M, Hata T, Takemasa I, Mizushima T, Soh JW, Doki Y, Mori M, Yamamoto H. Concurrent Targeting of KRAS and AKT by MiR-4689 Is a Novel Treatment Against Mutant KRAS Colorectal Cancer. *Mol Ther Nucleic Acids* 2015; **4**: e231 [PMID: 25756961 DOI: 10.1038/mtna.2015.5]

136 **Fang L**, Li H, Wang L, Hu J, Jin T, Wang J, Yang BB. MicroRNA-17-5p promotes chemotherapeutic drug resistance and tumour metastasis of colorectal cancer by repressing PTEN expression. *Oncotarget* 2014; **5**: 2974-2987 [PMID: 24912422 DOI: 10.18632/oncotarget.1614]

137 **Wu W**, Yang J, Feng X, Wang H, Ye S, Yang P, Tan W, Wei G, Zhou Y. MicroRNA-32 (miR-32) regulates phosphatase and tensin homologue (PTEN) expression and promotes growth, migration,

and invasion in colorectal carcinoma cells. *Mol Cancer* 2013; **12**: 30 [PMID: 23617834 DOI: 10.1186/1476-4598-12-30]

138 **Chen ML**, Liang LS, Wang XK. miR-200c inhibits invasion and migration in human colon cancer cells SW480/620 by targeting ZEB1. *Clin Exp Metastasis* 2012; **29**: 457-469 [PMID: 22407310 DOI: 10.1007/s10585-012-9463-7]

139 **Zaravinos A**. The Regulatory Role of MicroRNAs in EMT and Cancer. *J Oncol* 2015; **2015**: 865816 [PMID: 25883654 DOI: 10.1155/2015/865816]

140 **Wang J**, Du Y, Liu X, Cho WC, Yang Y. MicroRNAs as Regulator of Signaling Networks in Metastatic Colon Cancer. *Biomed Res Int* 2015; **2015**: 823620 [PMID: 26064956 DOI: 10.1155/2015/823620]

141 **Bu P**, Wang L, Chen KY, Rakhilin N, Sun J, Closa A, Tung KL, King S, Kristine Varanko A, Xu Y, Huan Chen J, Zessin AS, Shealy J, Cummings B, Hsu D, Lipkin SM, Moreno V, Gümüş ZH, Shen X. miR-1269 promotes metastasis and forms a positive feedback loop with TGF-β. *Nat Commun* 2015; **6**: 6879 [PMID: 25872451 DOI: 10.1038/ncomms7879]

142 **Loupakis F**, Cremolini C, Masi G, Lonardi S, Zagonel V, Salvatore L, Cortesi E, Tomasello G, Ronzoni M, Spadi R, Zaniboni A, Tonini G, Buonadonna A, Amoroso D, Chiara S, Carlomagno C, Boni C, Allegrini G, Boni L, Falcone A. Initial therapy with FOLFOXIRI and bevacizumab for metastatic colorectal cancer. *N Engl J Med* 2014; **371**: 1609-1618 [PMID: 25337750 DOI: 10.1056/NEJMoa1403108]

143 **Hu T**, Li Z, Gao CY, Cho CH. Mechanisms of drug resistance in colon cancer and its therapeutic strategies. *World J Gastroenterol* 2016; **22**: 6876-6889 [PMID: 27570424 DOI: 10.3748/wjg.vss.i30.6876]

144 **Amirkhah R**, Farazmand A, Irfan-Maqsood M, Wolkenhauer O, Schmitz U. The role of microRNAs in the resistance to colorectal cancer treatments. *Cell Mol Biol* (Noisy-le-grand) 2015; **61**: 17-23 [PMID: 26518892]

145 **Xie T**, Huang M, Wang Y, Wang L, Chen C, Chu X. MicroRNAs as Regulators, Biomarkers and Therapeutic Targets in the Drug Resistance of Colorectal Cancer. *Cell Physiol Biochem* 2016; **40**: 62-76 [PMID: 27842308 DOI: 10.1159/000452525]

146 **Wu QB**, Sheng X, Zhang N, Yang MW, Wang F. Role of microRNAs in the resistance of colorectal cancer to chemoradiotherapy. *Mol Clin Oncol* 2018; **8**: 528-532 [PMID: 29556386 DOI: 10.3892/mco.2018.1578]

147 **Nijhuis A**, Thompson H, Adam J, Parker A, Gammon L, Lewis A, Bundy JG, Soga T, Jalaly A, Propper D, Jeffery R, Suraweera N, McDonald S, Thaha MA, Feakins R, Lowe R, Bishop CL, Silver A. Remodelling of microRNAs in colorectal cancer by hypoxia alters metabolism profiles and 5-fluorouracil resistance. *Hum Mol Genet* 2017; **26**: 1552-1564 [PMID: 28207045 DOI: 10.1093/hmg/ddx059]

148 **Akao Y**, Noguchi S, Iio A, Kojima K, Takagi T, Naoe T. Dysregulation of microRNA-34a expression causes drug-resistance to 5-FU in human colon cancer DLD-1 cells. *Cancer Lett* 2011; **300**: 197-204 [PMID: 21067862 DOI: 10.1016/j.canlet.2010.10.006]

149 **Shang J**, Yang F, Wang Y, Wang Y, Xue G, Mei Q, Wang F, Sun S. MicroRNA-23a antisense enhances 5-fluorouracil chemosensitivity through APAF-1/caspase-9 apoptotic pathway in colorectal cancer cells. *J Cell Biochem* 2014; **115**: 772-784 [PMID: 24249161 DOI: 10.1002/jcb.24721]

150 **Chai J**, Dong W, Xie C, Wang L, Han DL, Wang S, Guo HL, Zhang ZL. MicroRNA-494 sensitizes colon cancer cells to fluorouracil through regulation of DPYD. *IUBMB Life* 2015; **67**: 191-201 [PMID: 25873402 DOI: 10.1002/iub.1361]

151 **Zhang Y**, Talmon G, Wang J. MicroRNA-587 antagonizes 5-FU-induced apoptosis and confers drug resistance by regulating PPP2R1B expression in colorectal cancer. *Cell Death Dis* 2015; **6**: e1845 [PMID: 26247730 DOI: 10.1038/cddis.2015.200]

152 **To KK**, Robey RW, Knutsen T, Zhan Z, Ried T, Bates SE. Escape

from hsa-miR-519c enables drug-resistant cells to maintain high expression of ABCG2. *Mol Cancer Ther* 2009; **8**: 2959-2968 [PMID: 19825807 DOI: 10.1158/1535-7163.MCT-09-0292]

153 **To KK**, Leung WW, Ng SS. Exploiting a novel miR-519c-HuR-ABCG2 regulatory pathway to overcome chemoresistance in colorectal cancer. *Exp Cell Res* 2015; **338**: 222-231 [PMID: 26386386 DOI: 10.1016/j.yexcr.2015.09.011]

154 **Zhou Y**, Wan G, Spizzo R, Ivan C, Mathur R, Hu X, Ye X, Lu J, Fan F, Xia L, Calin GA, Ellis LM, Lu X. miR-203 induces oxaliplatin resistance in colorectal cancer cells by negatively regulating ATM kinase. *Mol Oncol* 2014; **8**: 83-92 [PMID: 24145123 DOI: 10.1016/j.molonc.2013.09.004]

155 **Xu K**, Liang X, Cui D, Wu Y, Shi W, Liu J. miR-1915 inhibits Bcl-2 to modulate multidrug resistance by increasing drug-sensitivity in human colorectal carcinoma cells. *Mol Carcinog* 2013; **52**: 70-78 [PMID: 22121083 DOI: 10.1002/mc.21832]

156 **Bitarte N**, Bandres E, Boni V, Zarate R, Rodriguez J, Gonzalez-Huarriz M, Lopez I, Javier Sola J, Alonso MM, Fortes P, Garcia-Foncillas J. MicroRNA-451 is involved in the self-renewal, tumorigenicity, and chemoresistance of colorectal cancer stem cells. *Stem Cells* 2011; **29**: 1661-1671 [PMID: 21948564 DOI: 10.1002/stem.741]

157 **Chen Z**, Gao S, Wang D, Song D, Feng Y. Colorectal cancer cells are resistant to anti-EGFR monoclonal antibody through adapted autophagy. *Am J Transl Res* 2016; **8**: 1190-1196 [PMID: 27158405]

158 **Rupaimoole R**, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discov* 2017; **16**: 203-222 [PMID: 28209991 DOI: 10.1038/nrd.2016.246]

159 **Christopher AF**, Kaur RP, Kaur G, Kaur A, Gupta V, Bansal P. MicroRNA therapeutics: Discovering novel targets and developing specific therapy. *Perspect Clin Res* 2016; **7**: 68-74 [PMID: 27141472 DOI: 10.4103/2229-3485.179431]

160 **van Rooij E**, Kauppinen S. Development of microRNA therapeutics is coming of age. *EMBO Mol Med* 2014; **6**: 851-864 [PMID: 24935956 DOI: 10.15252/emmm.201100899]

161 **Thorsen SB**, Obad S, Jensen NF, Stenvang J, Kauppinen S. The therapeutic potential of microRNAs in cancer. *Cancer J* 2012; **18**: 275-284 [PMID: 22647365 DOI: 10.1097/PPO.0b013e318258b5d6]

162 **Bader AG**. miR-34 - a microRNA replacement therapy is headed to the clinic. *Front Genet* 2012; **3**: 120 [PMID: 22783274 DOI: 10.3389/fgene.2012.00120]

163 **Ibrahim AF**, Weirauch U, Thomas M, Grünweller A, Hartmann RK, Aigner A. MicroRNA replacement therapy for miR-145 and miR-33a is efficacious in a model of colon carcinoma. *Cancer Res* 2011; **71**: 5214-5224 [PMID: 21690566 DOI: 10.1158/0008-5472.CAN-10-4645]

164 **Liang G**, Zhu Y, Jing A, Wang J, Hu F, Feng W, Xiao Z, Chen B. Cationic microRNA-delivering nanocarriers for efficient treatment of colon carcinoma in xenograft model. *Gene Ther* 2016; **23**: 829-838 [PMID: 27482839 DOI: 10.1038/gt.2016.60]

165 **Adams BD**, Parsons C, Slack FJ. The tumor-suppressive and potential therapeutic functions of miR-34a in epithelial carcinomas. *Expert Opin Ther Targets* 2016; **20**: 737-753 [PMID: 26652031 DOI: 10.1517/14728222.2016.1114102]

166 **Beg MS**, Brenner AJ, Sachdev J, Borad M, Kang YK, Stoudemire J, Smith S, Bader AG, Kim S, Hong DS. Phase I study of MRX34, a liposomal miR-34a mimic, administered twice weekly in patients with advanced solid tumors. *Invest New Drugs* 2017; **35**: 180-188 [PMID: 27917453 DOI: 10.1007/s10637-016-0407-y]

167 **Garzon R**, Marcucci G, Croce CM. Targeting microRNAs in cancer: rationale, strategies and challenges. *Nat Rev Drug Discov* 2010; **9**: 775-789 [PMID: 20885409 DOI: 10.1038/nrd3179]

168 **Wu W**, Wang Z, Yang P, Yang J, Liang J, Chen Y, Wang H, Wei G, Ye S, Zhou Y. MicroRNA-135b regulates metastasis suppressor 1 expression and promotes migration and invasion in colorectal

cancer. *Mol Cell Biochem* 2014; **388**: 249-259 [PMID: 24343340 DOI: 10.1007/s11010-013-1916-z]

169　**Huang Z**, Huang S, Wang Q, Liang L, Ni S, Wang L, Sheng W, He X, Du X. MicroRNA-95 promotes cell proliferation and targets sorting Nexin 1 in human colorectal carcinoma. *Cancer Res* 2011; **71**: 2582-2589 [PMID: 21427358 DOI: 10.1158/0008-5472.CAN-10-3032]

170　**Chai H**, Liu M, Tian R, Li X, Tang H. miR-20a targets BNIP2 and contributes chemotherapeutic resistance in colorectal adenocarcinoma SW480 and SW620 cell lines. *Acta Biochim Biophys Sin* (Shanghai) 2011; **43**: 217-225 [PMID: 21242194 DOI: 10.1093/abbs/gmg125]

171　**Tsang WP**, Ng EK, Ng SS, Jin H, Yu J, Sung JJ, Kwok TT. Oncofetal H19-derived miR-675 regulates tumor suppressor RB in human colorectal cancer. *Carcinogenesis* 2010; **31**: 350-358 [PMID: 19926638 DOI: 10.1093/carcin/bgp181]

172　**Hagedorn PH**, Persson R, Funder ED, Albæk N, Diemer SL, Hansen DJ, Møller MR, Papargyri N, Christiansen H, Hansen BR, Hansen HF, Jensen MA, Koch T. Locked nucleic acid: modality, diversity, and drug discovery. *Drug Discov Today* 2018; **23**: 101-114 [PMID: 28988994 DOI: 10.1016/j.drudis.2017.09.018]

173　**Vester B**, Wengel J. LNA (locked nucleic acid): high-affinity targeting of complementary RNA and DNA. *Biochemistry* 2004; **43**: 13233-13241 [PMID: 15491130 DOI: 10.1021/bi0485732]

174　**You Y**, Moreira BG, Behlke MA, Owczarzy R. Design of LNA probes that improve mismatch discrimination. *Nucleic Acids Res* 2006; **34**: e60 [PMID: 16670427 DOI: 10.1093/nar/gkl175]

175　**Nedaeinia R**, Sharifi M, Avan A, Kazemi M, Rafiee L, Ghayour-Mobarhan M, Salehi R. Locked nucleic acid anti-miR-21 inhibits cell growth and invasive behaviors of a colorectal adenocarcinoma cell line: LNA-anti-miR as a novel approach. *Cancer Gene Ther* 2016; **23**: 246-253 [PMID: 27364574 DOI: 10.1038/cgt.2016.25]

176　**Choi WY**, Giraldez AJ, Schier AF. Target protectors reveal dampening and balancing of Nodal agonist and antagonist by miR-430. *Science* 2007; **318**: 271-274 [PMID: 17761850 DOI: 10.1126/science.1147535]

177　**Bu P**, Chen KY, Chen JH, Wang L, Walters J, Shin YJ, Goerger JP, Sun J, Witherspoon M, Rakhilin N, Li J, Yang H, Milsom J, Lee S, Zipfel W, Jin MM, Gümüş ZH, Lipkin SM, Shen X. A microRNA miR-34a-regulated bimodal switch targets Notch in colon cancer stem cells. *Cell Stem Cell* 2013; **12**: 602-615 [PMID: 23642368 DOI: 10.1016/j.stem.2013.03.002]

178　**Avitabile C**, Fabbri E, Bianchi N, Gambari R, Romanelli A. Inhibition of miRNA maturation by peptide nucleic acids. *Methods Mol Biol* 2014; **1095**: 157-164 [PMID: 24166311 DOI: 10.1007/978-1-62703-703-7]

179　**Gumireddy K**, Young DD, Xiong X, Hogenesch JB, Huang Q, Deiters A. Small-molecule inhibitors of microrna miR-21 function. *Angew Chem Int Ed Engl* 2008; **47**: 7482-7484 [PMID: 18712719 DOI: 10.1002/anie.200801555]

180　**Cruz-Gil S**, Sanchez-Martinez R, Gomez de Cedron M, Martin-Hernandez R, Vargas T, Molina S, Herranz J, Davalos A, Reglero G, Ramirez de Molina A. Targeting the lipid metabolic axis <i>ACSL/SCD</i> in colorectal cancer progression by therapeutic miRNAs: miR-19b-1 role. *J Lipid Res* 2018; **59**: 14-24 [PMID: 29074607 DOI: 10.1194/jlr.M076752]

181　**Sun RP**, Xi QY, Sun JJ, Cheng X, Zhu YL, Ye DZ, Chen T, Wei LM, Ye RS, Jiang QY, Zhang YL. In low protein diets, microRNA-19b regulates urea synthesis by targeting SIRT5. *Sci Rep* 2016; **6**: 33291 [PMID: 27686746 DOI: 10.1038/srep33291]

182　**Jarry J**, Schadendorf D, Greenwood C, Spatz A, van Kempen LC. The validity of circulating microRNAs in oncology: five years of challenges and contradictions. *Mol Oncol* 2014; **8**: 819-829 [PMID: 24656978 DOI: 10.1016/j.molonc.2014.02.009]

183　**Xiao G**, Tang H, Wei W, Li J, Ji L, Ge J. Aberrant Expression of MicroRNA-15a and MicroRNA-16 Synergistically Associates with Tumor Progression and Prognosis in Patients with Colorectal Cancer. *Gastroenterol Res Pract* 2014; **2014**: 364549 [PMID: 25435873 DOI: 10.1155/2014/364549]

184　**Díaz R**, Silva J, García JM, Lorenzo Y, García V, Peña C, Rodríguez R, Muñoz C, García F, Bonilla F, Domínguez G. Deregulated expression of miR-106a predicts survival in human colon cancer patients. *Genes Chromosomes Cancer* 2008; **47**: 794-802 [PMID: 18521848 DOI: 10.1002/gcc.20580]

185　**Gao J**, Li N, Dong Y, Li S, Xu L, Li X, Li Y, Li Z, Ng SS, Sung JJ, Shen L, Yu J. miR-34a-5p suppresses colorectal cancer metastasis and predicts recurrence in patients with stage II/III colorectal cancer. *Oncogene* 2015; **34**: 4142-4152 [PMID: 25362853 DOI: 10.1038/onc/2014.348]

186　**Mokutani Y**, Uemura M, Munakata K, Okuzaki D, Haraguchi N, Takahashi H, Nishimura J, Hata T, Murata K, Takemasa I, Mizushima T, Doki Y, Mori M, Yamamoto H. Down-Regulation of microRNA-132 is Associated with Poor Prognosis of Colorectal Cancer. *Ann Surg Oncol* 2016; **23**: 599-608 [PMID: 26868958 DOI: 10.1245/s10434-016-5133-3]

187　**Nishimura J**, Handa R, Yamamoto H, Tanaka F, Shibata K, Mimori K, Takemasa I, Mizushima T, Ikeda M, Sekimoto M, Ishii H, Doki Y, Mori M. microRNA-181a is associated with poor prognosis of colorectal cancer. *Oncol Rep* 2012; **28**: 2221-2226 [PMID: 23023298 DOI: 10.3892/or.2012.2059]

188　**Bovell LC**, Shanmugam C, Putcha BD, Katkoori VR, Zhang B, Bae S, Singh KP, Grizzle WE, Manne U. The prognostic value of microRNAs varies with patient race/ethnicity and stage of colorectal cancer. *Clin Cancer Res* 2013; **19**: 3955-3965 [PMID: 23719259 DOI: 10.1158/1078-0432.CCR-12-3302]

189　**Wang X**, Wang J, Ma H, Zhang J, Zhou X. Downregulation of miR-195 correlates with lymph node metastasis and poor prognosis in colorectal cancer. *Med Oncol* 2012; **29**: 919-927 [PMID: 21390519 DOI: 10.1007/s12032-011-9880-5]

190　**Shen ZL**, Wang B, Jiang KW, Ye CX, Cheng C, Yan YC, Zhang JZ, Yang Y, Gao ZD, Ye YJ, Wang S. Downregulation of miR-199b is associated with distant metastasis in colorectal cancer via activation of SIRT1 and inhibition of CREB/KISS1 signaling. *Oncotarget* 2016; **7**: 35092-35105 [PMID: 27145368 DOI: 10.18632/oncotarget.9402]

191　**Karaayvaz M**, Pal T, Song B, Zhang C, Georgakopoulos P, Mehmood S, Burke S, Shroyer K, Ju J. Prognostic significance of miR-215 in colon cancer. *Clin Colorectal Cancer* 2011; **10**: 340-347 [PMID: 21752725 DOI: 10.1016/j.clcc.2011.06.002]

192　**Li PL**, Zhang X, Wang LL, Du LT, Yang YM, Li J, Wang CX. MicroRNA-218 is a prognostic indicator in colorectal cancer and enhances 5-fluorouracil-induced apoptosis by targeting BIRC5. *Carcinogenesis* 2015; **36**: 1484-1493 [PMID: 26442524 DOI: 10.1093/carcin/bgv145]

193　**Dong SJ**, Cai XJ, Li SJ. The Clinical Significance of MiR-429 as a Predictive Biomarker in Colorectal Cancer Patients Receiving 5-Fluorouracil Treatment. *Med Sci Monit* 2016; **22**: 3352-3361 [PMID: 27654003]

194　**Yang IP**, Tsai HL, Miao ZF, Huang CW, Kuo CH, Wu JY, Wang WM, Juo SH, Wang JY. Development of a deregulating microRNA panel for the detection of early relapse in postoperative colorectal cancer patients. *J Transl Med* 2016; **14**: 108 [PMID: 27126129 DOI: 10.1186/s12967-016-0856-2]

195　**Coebergh van den Braak RRJ**, Sieuwerts AM, Lalmahomed ZS, Smid M, Wilting SM, Bril SI, Xiang S, van der Vlugt-Daane M, de Weerd V, van Galen A, Biermann K, van Krieken JHJM, Kloosterman WP, Foekens JA; MATCH study group*, Martens JWM, IJzermans JNM. Confirmation of a metastasis-specific microRNA signature in primary colon cancer. *Sci Rep* 2018; **8**: 5242 [PMID: 29588449 DOI: 10.1038/s41598-018-22532-1]

196　**Xiong Y**, Wang R, Peng L, You W, Wei J, Zhang S, Wu X, Guo J, Xu J, Lv Z, Fu Z. An integrated lncRNA, microRNA and mRNA signature to improve prognosis prediction of colorectal cancer. *Oncotarget* 2017; **8**: 85463-85478 [PMID: 29156733 DOI: 10.18632/oncotarget.20013]

197　**Menéndez P**, Padilla D, Villarejo P, Palomino T, Nieto P,

Menéndez JM, Rodríguez-Montes JA. Prognostic implications of serum microRNA-21 in colorectal cancer. *J Surg Oncol* 2013; **108**: 369-373 [PMID: 23970420 DOI: 10.1002/jso.23415]

198 **Kou CH**, Zhou T, Han XL, Zhuang HJ, Qian HX. Downregulation of mir-23b in plasma is associated with poor prognosis in patients with colorectal cancer. *Oncol Lett* 2016; **12**: 4838-4844 [PMID: 28101227 DOI: 10.3892/ol.2016.5265]

199 **Sun Y**, Liu Y, Cogdell D, Calin GA, Sun B, Kopetz S, Hamilton SR, Zhang W. Examining plasma microRNA markers for colorectal cancer at different stages. *Oncotarget* 2016; **7**: 11434-11449 [PMID: 26863633 DOI: 10.18632/oncotarget.7196]

200 **Jinushi T**, Shibayama Y, Kinoshita I, Oizumi S, Jinushi M, Aota T, Takahashi T, Horita S, Dosaka-Akita H, Iseki K. Low expression levels of microRNA-124-5p correlated with poor prognosis in colorectal cancer via targeting of SMC4. *Cancer Med* 2014; **3**: 1544-1552 [PMID: 25081869 DOI: 10.1002/cam4.309]

201 **Tsai HL**, Yang IP, Huang CW, Ma CJ, Kuo CH, Lu CY, Juo SH, Wang JY. Clinical significance of microRNA-148a in patients with early relapse of stage II stage and III colorectal cancer after curative resection. *Transl Res* 2013; **162**: 258-268 [PMID: 23933284 DOI: 10.1016/j.trsl.2013.07.009]

202 **Lv ZC**, Fan YS, Chen HB, Zhao DW. Investigation of microRNA-155 as a serum diagnostic and prognostic biomarker for colorectal cancer. *Tumour Biol* 2015; **36**: 1619-1625 [PMID: 25528214 DOI: 10.1007/s13277-014-2760-9]

203 **Yuan D**, Li K, Zhu K, Yan R, Dang C. Plasma miR-183 predicts recurrence and prognosis in patients with colorectal cancer. *Cancer Biol Ther* 2015; **16**: 268-275 [PMID: 25629978 DOI: 10.1080/15384047.2014.1002327]

204 **Toiyama Y**, Hur K, Tanaka K, Inoue Y, Kusunoki M, Boland CR, Goel A. Serum miR-200c is a novel prognostic and metastasis-predictive biomarker in patients with colorectal cancer. *Ann Surg* 2014; **259**: 735-743 [PMID: 23982750 DOI: 10.1097/SLA.0b013e3182a6909d]

205 **Hur K**, Toiyama Y, Okugawa Y, Ide S, Imaoka H, Boland CR, Goel A. Circulating microRNA-203 predicts prognosis and metastasis in human colorectal cancer. *Gut* 2017; **66**: 654-665 [PMID: 26701878 DOI: 10.1136/gutjnl-2014-308737]

206 **Yu H**, Gao G, Jiang L, Guo L, Lin M, Jiao X, Jia W, Huang J. Decreased expression of miR-218 is associated with poor prognosis in patients with colorectal cancer. *Int J Clin Exp Pathol* 2013; **6**: 2904-2911 [PMID: 24294377]

207 **Pu XX**, Huang GL, Guo HQ, Guo CC, Li H, Ye S, Ling S, Jiang L, Tian Y, Lin TY. Circulating miR-221 directly amplified from plasma is a potential diagnostic and prognostic marker of colorectal cancer and is correlated with p53 expression. *J Gastroenterol Hepatol* 2010; **25**: 1674-1680 [PMID: 20880178 DOI: 10.1111/j.1440-1746.2010.06417.x]

208 **Hur K**, Toiyama Y, Schetter AJ, Okugawa Y, Harris CC, Boland CR, Goel A. Identification of a metastasis-specific MicroRNA signature in human colorectal cancer. *J Natl Cancer Inst* 2015; **107**: pii: dju492 [PMID: 25663689 DOI: 10.1093/jnci/dju492]

209 **Imaoka H**, Toiyama Y, Fujikawa H, Hiro J, Saigusa S, Tanaka K, Inoue Y, Mohri Y, Mori T, Kato T, Toden S, Goel A, Kusunoki M. Circulating microRNA-1290 as a novel diagnostic and prognostic biomarker in human colorectal cancer. *Ann Oncol* 2016; **27**: 1879-1886 [PMID: 27502702 DOI: 10.1093/annonc/mdw279]

210 **Vychytilova-Faltejskova P**, Radova L, Sachlova M, Kosarova Z, Slaba K, Fabian P, Grolich T, Prochazka V, Kala Z, Svoboda M, Kiss I, Vyzula R, Slaby O. Serum-based microRNA signatures in early diagnosis and prognosis prediction of colon cancer. *Carcinogenesis* 2016; **37**: 941-950 [PMID: 27485599 DOI: 10.1093/carcin/bgw078]

211 **Ding M**, Zhang T, Li S, Zhang Y, Qiu Y, Zhang B. Correlation analysis between liver metastasis and serum levels of miR-200 and miR-141 in patients with colorectal cancer. *Mol Med Rep* 2017; **16**: 7791-7795 [PMID: 28944840 DOI: 10.3892/mmr.2017.7538]

212 **Maierthaler M**, Benner A, Hoffmeister M, Surowy H, Jansen

L, Knebel P, Chang-Claude J, Brenner H, Burwinkel B. Plasma miR-122 and miR-200 family are prognostic markers in colorectal cancer. *Int J Cancer* 2017; **140**: 176-187 [PMID: 27632639 DOI: 10.1002/ijc.30433]

213 **Rotelli MT**, Di Lena M, Cavallini A, Lippolis C, Bonfrate L, Chetta N, Portincasa P, Altomare DF. Fecal microRNA profile in patients with colorectal carcinoma before and after curative surgery. *Int J Colorectal Dis* 2015; **30**: 891-898 [PMID: 25989926 DOI: 10.1007/s00384-015-2248-0]

214 **Ogata-Kawata H**, Izumiya M, Kurioka D, Honma Y, Yamada Y, Furuta K, Gunji T, Ohta H, Okamoto H, Sonoda H, Watanabe M, Nakagama H, Yokota J, Kohno T, Tsuchiya N. Circulating exosomal microRNAs as biomarkers of colon cancer. *PLoS One* 2014; **9**: e92921 [PMID: 24705249 DOI: 10.1371/journal.pone.0092921]

215 **Takano Y**, Masuda T, Iinuma H, Yamaguchi R, Sato K, Tobo T, Hirata H, Kuroda Y, Nambara S, Hayashi N, Iguchi T, Ito S, Eguchi H, Ochiya T, Yanaga K, Miyano S, Mimori K. Circulating exosomal microRNA-203 is associated with metastasis possibly via inducing tumor-associated macrophages in colorectal cancer. *Oncotarget* 2017; **8**: 78598-78613 [PMID: 29108252 DOI: 10.18632/omcotarget.20009]

216 **Yan S**, Han B, Gao S, Wang X, Wang Z, Wang F, Zhang J, Xu D, Sun B. Exosome-encapsulated microRNAs as circulating biomarkers for colorectal cancer. *Oncotarget* 2017; **8**: 60149-60158 [PMID: 28947960 DOI: 10.18632/oncotarget.18557]

217 **Li J**, Li B, Ren C, Chen Y, Guo X, Zhou L, Peng Z, Tang Y, Chen Y, Liu W, Zhu B, Wang L, Liu X, Shi X, Peng Z. The clinical significance of circulating GPC1 positive exosomes and its regulative miRNAs in colon cancer patients. *Oncotarget* 2017; **8**: 101189-101202 [PMID: 29254156 DOI: 10.18632/oncotarget.20516]

218 **Cha DJ**, Franklin JL, Dou Y, Liu Q, Higginbotham JN, Demory Beckler M, Weaver AM, Vickers K, Prasad N, Levy S, Zhang B, Coffey RJ, Patton JG. KRAS-dependent sorting of miRNA to exosomes. *Elife* 2015; **4**: e07197 [PMID: 26132860 DOI: 10.7554/eLife07197]

219 219 **Chiba M**, Kimura M, Asari S. Exosomes secreted from human colorectal cancer cell lines contain mRNAs, microRNAs and natural antisense RNAs, that can transfer into the human hepatoma HepG2 and lung cancer A549 cell lines. *Oncol Rep* 2012; **28**: 1551-1558 [PMID: 22895844 DOI: 10.3892/or.2012.1967]

220 **Bigagli E**, Luceri C, Guasti D, Cinci L. Exosomes secreted from human colon cancer cells influence the adhesion of neighboring metastatic cells: Role of microRNA-210. *Cancer Biol Ther* 2016; 1-8 [PMID: 27611932 DOI: 10.1080/15384047.2016.1219815]

221 **Clancy C**, Khan S, Glynn CL, Holian E, Dockery P, Lalor P, Brown JA, Joyce MR, Kerin MJ, Dwyer RM. Screening of exosomal microRNAs from colorectal cancer cells. *Cancer Biomark* 2016; **17**: 427-435 [PMID: 27802194 DOI: 10.3233/CBM-160659]

222 **Lopes-Ramos CM**, Habr-Gama A, Quevedo Bde S, Felício NM, Bettoni F, Koyama FC, Asprino PF, Galante PA, Gama-Rodrigues J, Camargo AA, Perez RO, Parmigiani RB. Overexpression of miR-21-5p as a predictive marker for complete tumor regression to neoadjuvant chemoradiotherapy in rectal cancer patients. *BMC Med Genomics* 2014; **7**: 68 [PMID: 25496125 DOI: 10.1186/s12920-014-0068-7]

223 **Ragusa M**, Majorana A, Statello L, Maugeri M, Salito L, Barbagallo D, Guglielmino MR, Duro LR, Angelica R, Caltabiano R, Biondi A, Di Vita M, Privitera G, Scalia M, Cappellani A, Vasquez E, Lanzafame S, Basile F, Di Pietro C, Purrello M. Specific alterations of microRNA transcriptome and global network structure in colorectal carcinoma after cetuximab treatment. *Mol Cancer Ther* 2010; **9**: 3396-3409 [PMID: 20881268 DOI: 10.1158/1535-7163.MCT-10-0137]

224 **Boisen MK**, Dehlendorff C, Linnemann D, Nielsen BS, Larsen JS, Osterlind K, Nielsen SE, Tarpgaard LS, Qvortrup C, Pfeiffer P, Holländer NH, Keldsen N, Hansen TF, Jensen BB, Høgdall

EV, Jensen BV, Johansen JS. Tissue microRNAs as predictors of outcome in patients with metastatic colorectal cancer treated with first line Capecitabine and Oxaliplatin with or without Bevacizumab. *PLoS One* 2014; **9**: e109430 [PMID: 25329796 DOI: 10.1371/journal.pone.0109430]

225 **Molina-Pinelo S**, Carnero A, Rivera F, Estevez-Garcia P, Bozada JM, Limon ML, Benavent M, Gomez J, Pastor MD, Chaves M, Suarez R, Paz-Ares L, de la Portilla F, Carranza-Carranza A, Sevilla I, Vicioso L, Garcia-Carbonero R. MiR-107 and miR-99a-3p predict chemotherapy response in patients with advanced colorectal cancer. *BMC Cancer* 2014; **14**: 656 [PMID: 25197016 DOI: 10.1186/1471-2407-14-656]

226 **Chen Q**, Xia HW, Ge XJ, Zhang YC, Tang QL, Bi F. Serum miR-19a predicts resistance to FOLFOX chemotherapy in advanced colorectal cancer cases. *Asian Pac J Cancer Prev* 2013; **14**: 7421-7426 [PMID: 24460313]

227 **Qian X**, Yu J, Yin Y, He J, Wang L, Li Q, Zhang LQ, Li CY, Shi ZM, Xu Q, Li W, Lai LH, Liu LZ, Jiang BH. MicroRNA-143 inhibits tumor growth and angiogenesis and sensitizes chemosensitivity to oxaliplatin in colorectal cancers. *Cell Cycle* 2013; **12**: 1385-1394 [PMID: 23574723 DOI: 10.4161/cc.24477]

228 **Hu J**, Cai G, Xu Y, Cai S. The Plasma microRNA miR-1914* and -1915 Suppresses Chemoresistant in Colorectal Cancer Patients by Down-regulating NFIX. *Curr Mol Med* 2016; **16**: 70-82 [PMID: 26695693]

229 **Nakajima G**, Hayashi K, Xi Y, Kudo K, Uchida K, Takasaki K, Yamamoto M, Ju J. Non-coding MicroRNAs hsa-let-7g and hsa-miR-181b are Associated with Chemoresponse to S-1 in Colon Cancer. *Cancer Genomics Proteomics* 2006; **3**: 317-324 [PMID: 18172508]

230 **Nishida N**, Yamashita S, Mimori K, Sudo T, Tanaka F, Shibata K, Yamamoto H, Ishii H, Doki Y, Mori M. MicroRNA-10b is a prognostic indicator in colorectal cancer and confers resistance to the chemotherapeutic agent 5-fluorouracil in colorectal cancer cells. *Ann Surg Oncol* 2012; **19**: 3065-3071 [PMID: 22322955 DOI: 10.1245/s10434-012-2246-1]

231 **Kurokawa K**, Tanahashi T, Iima T, Yamamoto Y, Akaike Y, Nishida K, Masuda K, Kuwano Y, Murakami Y, Fukushima M, Rokutan K. Role of miR-19b and its target mRNAs in 5-fluorouracil resistance in colon cancer cells. *J Gastroenterol* 2012; **47**: 883-895 [PMID: 22382630 DOI: 10.1007/s00535-012-0547-6]

232 **Wang CJ**, Stratmann J, Zhou ZG, Sun XF. Suppression of microRNA-31 increases sensitivity to 5-FU at an early stage, and affects cell migration and invasion in HCT-116 colon cancer cells. *BMC Cancer* 2010; **10**: 616 [PMID: 21062447 DOI: 10.1186/1471-2407-10-616]

233 **Song B**, Wang Y, Xi Y, Kudo K, Bruheim S, Botchkina GI, Gavin E, Wan Y, Formentini A, Kornmann M, Fodstad O, Ju J. Mechanism of chemoresistance mediated by miR-140 in human osteosarcoma and colon cancer cells. *Oncogene* 2009; **28**: 4065-4074 [PMID: 19734943 DOI: 10.1038/onc.2009.274]

234 **Song B**, Wang Y, Titmus MA, Botchkina G, Formentini A, Kornmann M, Ju J. Molecular mechanism of chemoresistance by miR-215 in osteosarcoma and colon cancer cells. *Mol Cancer* 2010; **9**: 96 [PMID: 20433742 DOI: 10.1186/1476-4598-9-96]

235 **Boni V**, Bitarte N, Cristobal I, Zarate R, Rodriguez J, Maiello E, Garcia-Foncillas J, Bandres E. miR-192/miR-215 influence 5-fluorouracil resistance through cell cycle-mediated mechanisms complementary to its post-transcriptional thymidilate synthase regulation. *Mol Cancer Ther* 2010; **9**: 2265-2275 [PMID: 20647341 DOI: 10.1158/1535-7163.MCT-10-0061]

236 **Kim C**, Hong Y, Lee H, Kang H, Lee EK. MicroRNA-195 desensitizes HCT116 human colon cancer cells to 5-fluorouracil. *Cancer Lett* 2018; **412**: 264-271 [PMID: 29080751 DOI: 10.1016/j.canlet.2017.10.022]

237 **Amankwatia EB**, Chakravarty P, Carey FA, Weidlich S, Steele RJ, Munro AJ, Wolf CR, Smith G. MicroRNA-224 is associated with colorectal cancer progression and response to 5-fluorouracil-based chemotherapy by KRAS-dependent and -independent mechanisms. *Br J Cancer* 2015; **112**: 1480-1490 [PMID: 25919696 DOI: 10.1038/bjc.2015.125]

238 **Zhang Y**, Geng L, Talmon G, Wang J. MicroRNA-520g confers drug resistance by regulating p21 expression in colorectal cancer. *J Biol Chem* 2015; **290**: 6215-6225 [PMID: 25616665 DOI: 10.1074/jbc.M114.620252]

239 **Li X**, Zhao H, Zhou X, Song L. Inhibition of lactate dehydrogenase A by microRNA-34a resensitizes colon cancer cells to 5-fluorouracil. *Mol Med Rep* 2015; **11**: 577-582 [PMID: 25333573 DOI: 10.3892/mmr.2014.2726]

240 **Akao Y**, Khoo F, Kumazaki M, Shinohara H, Miki K, Yamada N. Extracellular disposal of tumor-suppressor miRs-145 and -34a via microvesicles and 5-FU resistance of human colon cancer cells. *Int J Mol Sci* 2014; **15**: 1392-1401 [PMID: 24447928 DOI: 10.3390/ijms15011392]

241 **Sun C**, Wang FJ, Zhang HG, Xu XZ, Jia RC, Yao L, Qiao PF. miR-34a mediates oxaliplatin resistance of colorectal cancer cells by inhibiting macroautophagy via transforming growth factor-β/Smad4 pathway. *World J Gastroenterol* 2017; **23**: 1816-1827 [PMID: 28348487 DOI: 10.3748/wjg.v23.i10.1816]

242 **Liu H**, Yin Y, Hu Y, Feng Y, Bian Z, Yao S, Li M, You Q, Huang Z. miR-139-5p sensitizes colorectal cancer cells to 5-fluorouracil by targeting NOTCH-1. *Pathol Res Pract* 2016; **212**: 643-649 [PMID: 27173050 DOI: 10.1016/j.prp.2016.04.011]

243 **Zhang L**, Pickard K, Jenei V, Bullock MD, Bruce A, Mitter R, Kelly G, Paraskeva C, Strefford J, Primrose J, Thomas GJ, Packham G, Mirnezami AH. miR-153 supports colorectal cancer progression via pleiotropic effects that enhance invasion and chemotherapeutic resistance. *Cancer Res* 2013; **73**: 6435-6447 [PMID: 23950211 DOI: 10.1158/0008-5472.CAN-12-3308]

244 **Chang HY**, Ye SP, Pan SL, Kuo TT, Liu BC, Chen YL, Huang TC. Overexpression of miR-194 Reverses HMGA2-driven Signatures in Colorectal Cancer. *Theranostics* 2017; **7**: 3889-3900 [PMID: 29109785 DOI: 10.7150/thno.20041]

245 **Senfter D**, Holzner S, Kalipciyan M, Staribacher A, Walzl A, Huttary N, Krieger S, Brenner S, Jäger W, Krupitza G, Dolznig H, Mader RM. Loss of miR-200 family in 5-fluorouracil resistant colon cancer drives lymphendothelial invasiveness in vitro. *Hum Mol Genet* 2015; **24**: 3689-3698 [PMID: 25832648 DOI: 10.1093/hmg/ddv113]

246 **Lv Z**, Wei J, You W, Wang R, Shang J, Xiong Y, Yang H, Yang X, Fu Z. Disruption of the c-Myc/miR-200b-3p/PRDX2 regulatory loop enhances tumor metastasis and chemotherapeutic resistance in colorectal cancer. *J Transl Med* 2017; **15**: 257 [PMID: 29258530 DOI: 10.1186/s12967-017-1357-7]

247 **Li T**, Gao F, Zhang XP. miR-203 enhances chemosensitivity to 5-fluorouracil by targeting thymidylate synthase in colorectal cancer. *Oncol Rep* 2015; **33**: 607-614 [PMID: 25482885 DOI: 10.3892/or/2014.3646]

248 **Li P**, Zhang X, Wang H, Wang L, Liu T, Du L, Yang Y, Wang C. MALAT1 Is Associated with Poor Response to Oxaliplatin-Based Chemotherapy in Colorectal Cancer Patients and Promotes Chemoresistance through EZH2. *Mol Cancer Ther* 2017; **16**: 739-751 [PMID: 28069878 DOI: 10.1158/1535-7163.MCT-16-0591]

249 **Cao S**, Lin L, Xia X, Wu H. MicroRNA-761 promotes the sensitivity of colorectal cancer cells to 5-Fluorouracil through targeting FOXM1. *Oncotarget* 2017; **9**: 321-331 [PMID: 29416616 DOI: 10.18632/oncotarget.20109]

250 **Tanaka S**, Hosokawa M, Yonezawa T, Hayashi W, Ueda K, Iwakawa S. Induction of epithelial-mesenchymal transition and down-regulation of miR-200c and miR-141 in oxaliplatin-resistant colorectal cancer cells. *Biol Pharm Bull* 2015; **38**: 435-440 [PMID: 25757925 DOI: 10.1248/bpb.b14-00695]

251 **Yu T**, Guo F, Yu Y, Sun T, Ma D, Han J, Qian Y, Kryczek I, Sun D, Nagarsheth N, Chen Y, Chen H, Hong J, Zou W, Fang JY. Fusobacterium nucleatum Promotes Chemoresistance to Colorectal

Cancer by Modulating Autophagy. *Cell* 2017; **170**: 548-563.e16 [PMID: 28753429 DOI: 10.1016/j.cell.2017.07.008]

252 **Mlcochova J**, Faltejskova-Vychytilova P, Ferracin M, Zagatti B, Radova L, Svoboda M, Nemecek R, John S, Kiss I, Vyzula R, Negrini M, Slaby O. MicroRNA expression profiling identifies miR-31-5p/3p as associated with time to progression in wild-type RAS metastatic colorectal cancer treated with cetuximab. *Oncotarget* 2015; **6**: 38695-38704 [PMID: 26497852 DOI: 10.18632/oncotarget.5735]

253 **Lu Y**, Zhao X, Liu Q, Li C, Graves-Deal R, Cao Z, Singh B, Franklin JL, Wang J, Hu H, Wei T, Yang M, Yeatman TJ, Lee E, Saito-Diaz K, Hinger S, Patton JG, Chung CH, Emmrich S, Klusmann JH, Fan D, Coffey RJ. lncRNA MIR100HG-derived miR-100 and miR-125b mediate cetuximab resistance via Wnt/β-catenin signaling. *Nat Med* 2017; **23**: 1331-1341 [PMID: 29035371 DOI: 10.1038/nm.4424]

Pathogenesis of non-alcoholic fatty liver disease in children and adolescence: From "two hit theory" to "multiple hit model"

Yan-Lan Fang, Hong Chen, Chun-Lin Wang, Li Liang

Yan-Lan Fang, Chun-Lin Wang, Li Liang, Department of Pediatrics, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

Hong Chen, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

ORCID number: Yan-Lan Fang (0000-0001-6579-417X); Hong Chen (0000-0002-8678-2722); Chun-Lin Wang (0000-0002-8288-3075); Li Liang (0000-0002-2074-7406).

Abstract

Nonalcoholic fatty liver disease (NAFLD) has become the dominant form of chronic liver disease in children and adolescents with the increasing prevalence of obesity worldwide. NAFLD represents a wide spectrum of conditions, ranging from fatty liver - which generally follows a benign, non-progressive clinical course - to non-alcoholic steatohepatitis, a subset of NAFLD that may progress to cirrhosis and end-stage liver disease or liver carcinoma. The underlying pathophysiological mechanism of "pediatric" NAFLD remains unclear, although it is strongly associated with obesity and insulin resistance. In this review we provide a general overview on the current understanding of NAFLD in children and adolescents, which underpins practice, enabling early diagnosis and appropriate therapeutic intervention for this life-threatening liver disease.

Key words: Non-alcoholic steatohepatitis; Children; Adolescents; Pathogenesis; Nonalcoholic fatty liver disease

Core tip: Much work on nonalcoholic fatty liver disease (NAFLD) has been done, but an accurate understanding of its mechanism remains unclear. Our objective was to examine the current literature to better understand the

pathogenesis of NAFLD, thus showing how it evolved from the "two-hit theory" to a "multiple hit model".

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), the hepatic manifestation of the metabolic syndrome, is the most frequent form of chronic liver disease worldwide. Corresponding to abnormal fat accumulation in hepatocytes, it encompasses a spectrum of chronic liver diseases in the absence of excessive alcohol consumption, which may occur with or without hepatocyte inflammation or fibrosis[1]. Isolated steatosis, defined by abnormal accumulation of fat in more than 5% of hepatocytes, is a relatively benign condition.

In contrast, besides steatosis, non-alcoholic steatohepatitis (NASH) coexists with inflammation, hepatic cell injury, and deposition of collagen fibers[2]. NASH is a dynamic condition that can regress to isolated steatosis or cause progressive fibrosis leading to cirrhosis. The prevalence of NAFLD ranges from 10% to 30% depending on the study population and diagnostic methods used and is thought to be increasing worldwide[3,4]. Recently, a meta-analysis showed that the global prevalence of NAFLD is 25.24% (95%CI: 22.10%-28.65%) with the highest prevalence in the Middle East and South America and the lowest in Africa for the year 2016[5]. The "gold standard" test is liver biopsy, but this is neither feasible nor ethical for epidemiological studies aiming to screen NAFLD in the healthy population, and is also problematic in the clinical diagnosis. As so far, there was only one pediatric study which reported prevalence based on liver histology, and it reported that the prevalence of NAFLD in obese children (aged 2-19 years) was 38% and increased with age[6].

Serum biomarkers such as alanine aminotransferase (ALT) and aspartate aminotransferase, as well as liver imaging (liver ultrasound and magnetic resonance imaging), are currently the most widely used tools for screening. Published guidelines vary about the frequency of ALT screening and use of imaging[7-9]. Ongoing developments of new technologies are improving the diagnosis; however, the specificity and sensitivity for the diagnosis of NAFLD have not yet reached an acceptable level.

As mentioned above, NASH is progressive, so early diagnosis and treatments are critical. However, many aspects of the pathogenesis of NAFLD remain unclear; for example, the mechanism of the progression from steatosis to steatohepatitis. It is also unknown why NAFLD occurs only in a subgroup of obese subjects.

In the past decade, our team has researched the causes, pathogenesis, clinical diagnosis, and treatment of NAFLD[10-14]. In this review, we focus on the pathogenesis of NAFLD and explore new ways for improving both the diagnosis and treatment of NASH. The articles cited were identified based on a search of PubMed done in February 2018 using the criteria "NAFLD and pathogenesis and children and adolescent" with studies in humans and animals.

PATHOGENESIS OF NAFLD

Evolution from the "two hit theory" to the "multiple hit model"

During recent decades, the worldwide prevalence of obesity has increased in the pediatric population and the prevalence of NAFLD has more than doubled during the last 20 years in the United States[14]. The development of NAFLD is strongly influenced by age, sex, and ethnicity, and appears twice as often in boys than in girls[15-18]. NASH can progress to end-stage liver diseases such as hepatic cirrhosis or hepatocellular carcinoma. Conjeevaram PF *et al*[19] analyzed the database and discovered that as the prevalence of NAFLD increased, the prevalence of NASH also increased, however, compared to adult the prevalence of liver fibrosis in children remained low, which indicated a possibly less aggressive NAFLD phenotype in children. Although the prevalence of NAFLD is increasing, most affected patients present with isolated steatosis with only a minority of cases progressing to liver cirrhosis in children, and it is not clear whether pediatric and adult NAFLD are two different pathologic entities or just age-dependent manifestations of the same disease, which implies that the pathogenesis of NAFLD may be related to the interplay among genetic, environmental, and individual factors. Early theories of the pathogenesis of NAFLD and NASH were described in terms of the "two hit hypothesis"[20]. At the onset of disease, the "first hit" is represented by an increase in liver fat, characterized by hepatic triglyceride accumulation and insulin resistance, and corresponding to hepatic steatosis once the accumulation of hepatic fat is more than 5%. Children, especially pre-pubertal boys, have a pattern of type 2 NAFLD characterized by a zone 1 distribution of steatosis, inflammation and fibrosis[21]. Liver fat accumulation is associated with a hypercaloric diet, sedentary lifestyle, and is perhaps genetically predisposed. Our team successfully established an *in vivo* NAFLD animal model induced by a high-fat diet, and reported that lifestyle interventions have an effect on NAFLD in obese children[22,23]. Subsequently, the "second hit" emerges, which includes inflammatory cytokines, adipokines, mitochondrial dysfunction, and oxidative stress. As the fatty liver is more susceptible

to this "second hit", necroinflammation and fibrosis can develop and ultimately lead to cirrhosis[24,25]. However, with the development of new technology and further research, this view appears too simplistic for recapitulating the complexity of human NAFLD.

Now, the widely accepted theory is the "multiple-hit model", involving more widespread metabolic dysfunction because of the interaction of genetic and environmental factors as well as changes in crosstalk between different organs and tissues, including adipose tissue, the pancreas, gut, and liver[24-27]. However, liver fat accumulation, caused by obesity and insulin resistance, still seem to represent the "first hits".

MULTIPLE FACTORS

Fat accumulation and insulin resistance

Fat accumulates in the liver of patients with NAFLD mainly in the form of triglycerides, which derive from the esterification of glycerol and free fatty acids (FFAs)[28]. Triglyceride accumulation is not hepatotoxic, in contrast with the excess of FFAs that undergo acetyl coenzyme A (acyl-CoA) synthase activity and form fatty acyl-CoAs which may trigger esterification or β-oxidation pathways[29]. Mitochondrial dysfunction, which consists of oxidative stress and production of reactive oxygen species and endoplasmic reticulum stress-associated mechanisms, also results from NAFLD[30,31].

Physiologically, insulin controls hepatic glucose production by regulating lipolysis of adipocytes, leading to decreased fatty acid flux in the liver[32]. Consequently, the availability of hepatic acetyl coenzyme A (acyl-CoA) concentrations and the activity of pyruvate carboxylase are reduced, resulting in the decreased conversion of pyruvate to glucose (Figure 1).

Insulin resistance (IR) refers to a defective metabolic response to the effect of the hormone in the target cell (*e.g.*, muscle cell, hepatocyte, and adipocyte) or at the whole organism level. Systemic IR means that the ability of insulin to lower the serum glucose concentration to the appropriate level is hampered due to disrupted translocation of the GLUT4 receptor at the surface membrane of the muscle cell. As a result, glucose uptake (which depends on insulin) decreases. Hepatic IR consists of disturbed insulin mediated suppression of hepatic glucose production, but in the presence of preserved stimulation of lipogenesis[33]. In the adipose system, insulin resistance means that insulin is unable to suppress lipolysis. In humans, when the availability of lipids exceeds the lipid accumulation capacity, systemic IR and hepatic IR are likely to progress[34].

Inflammatory pathways

Increased FFA levels can cause lipotoxicity and insulin resistance, and together with other factors (such as gut-derived endotoxins), activate the release of proinflammatory cytokines systemically and also locally in the liver. There are two main classical pathways involved in the process of NAFLD inflammation: JNK-AP-1 and IKK-NF-κBD[35]. JNK-AP-1 is a mitogen-activated protein kinase associated with apoptosis and NASH; IKK-NF-κB is a transcription factor regulating inflammatory activation. Previous studies have shown that persistent activation of NF-κB was found in NAFLD animal models as well as in humans with NASH[36,37]. Animal models demonstrated that hepatic exposure to high levels of proinflammatory cytokines could lead to histological changes mimicking NASH[38].

The liver consists of parenchymal cells and nonparenchymal cells (NPCs); NPCs include sinusoidal endothelial cells. Kupffer cells (KCs) and hepatic stellate cells are less numerous than hepatocytes, but play a key role in the immune regulation of the liver, especially through substances released from KCs, which act as antigen presenting cells.

The hypothesis is that when the flow of FFAs or other pathogenic factors (such as endotoxins) from the gut into liver are excessive, KCs phagocytose the factors and present them through pattern recognition receptors (PRRs)[39]. PRRs include toll-like receptors (TLRS) such TLR4, TLR9, and nucleotide oligomerization domain-like receptors (NLRs)[40]. Inflammasomes, through NLR, activate the cascade events which finally generate mature IL-1, IL-8[41], and IL-1, contributing to regulate the activation of the transcription factor NF-κB[42]. KCs *per se* will differentiate into either the M1 or M2 phenotype, depending on the environmental inducer; the former releasing cytokines like TNF-α, IL-1, and IL-12 and the latter, more heterogeneous, being able to stimulate the secretion of IL-4, IL-10, and TGF-β according to different triggers[43]. IL-6 and TNF-α are the cytokines responsible for NASH progression. Patients with NASH have higher serum TNF-α levels, which play an important role in hepatic fibrosis through KC activation[38].

Therefore, TLR suppression is thought to block the immune response, thereby alleviating liver inflammation. However, to date, despite some animal experiments aiming to reveal the links between TLRs and NAFLD pathogenesis, no investigations on TLR agonists have yet been conducted in humans[44,45].

In summary, hepatocyte damage is an indicator of NASH progression. Different pathogens stimulate cell receptors thus activating the signaling pathway which contributes to cytokine production. Therefore, NASH might be detected at an earlier stage in the future by identifying an appropriate cytokine panel. Future studies should also focus on TLR modulation, which may provide a new target for NAFLD therapy.

Gut-liver axis

In recent years, many studies have been carried out on gut-liver axis (GLA) dysfunction (including intestinal dysbiosis, bacterial overgrowth, and alteration of mucosa permeability) intending to find the possible therapeutic target of NAFLD[46,47]. GLA is characterized by bidirectional traffic. Nutrients and factors derived
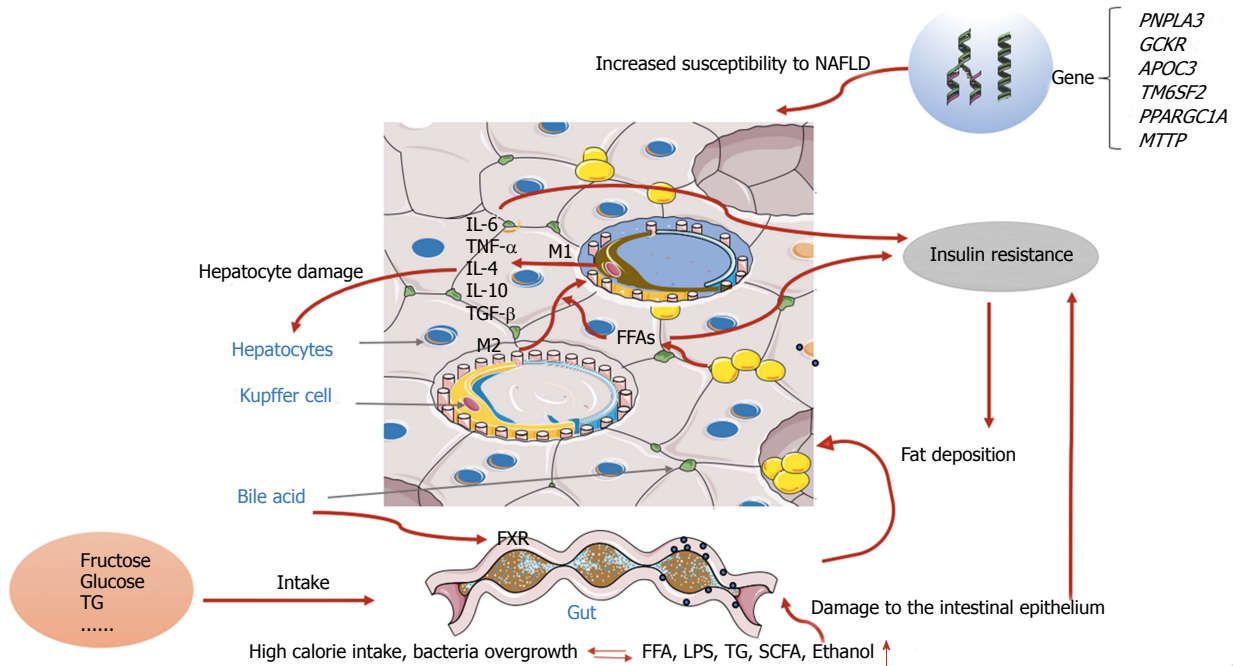
Figure 1 Schematic mechanistic diagram of the "multiple hit model". NAFLD: Nonalcoholic fatty liver disease.

from the gut lumen reach the liver through the portal circulation; bile acids, produced by hepatocytes, are released in the small intestine through the biliary tract[48]. Two of these components (intestinal barrier and gut microbiota) seem to play a key role in liver damage and its progression[49]. It is well known that trillions of microbes make up the gut microbiota. In normal conditions, only a small amount of bacteria products enter the liver through the portal circulation. However, bacteria dysbiosis or gut barrier alterations will increase the bacteria flow into the liver, thus stimulating inflammation *via* TLR and other pattern recognition receptor activation in KCs[50]. According to the bidirectional traffic of GLA, bile acid also impacts the gut environment, both directly by causing membrane damage and indirectly *via* the activation by bile acid metabolitas of special receptors such as the farnesoid X receptor. Gut microbiota (GM) is specific to each individual, but humans share a core functional microbiome[51].

Altered GM associated with NAFLD may occur through several mechanisms as follows: (1) GM digests and ferments the excessive energy into short-chain fatty acids (SCFAs); (2) GM bacteria can produce ethanol that may affect the liver in a similar way to chronic alcoholism; (3) bacteria/endotoxins translocate into the portal circulation and damage the liver *via* TLR signaling; and (4) disturbed lipid metabolism is mediated by increased bile acid synthesis and decreased choline metabolism[52].

In addition, GM also plays a vital role in maintaining gut barrier integrity and intestinal permeability. GM dysbiosis can damage the intestinal epithelium and destroy tight junction proteins, which is important in

preventing harmful substances from the gut such as bacteria, ethanol, and endotoxins from entering portal blood[46,53]. Experiments on mice and humans have confirmed these data[54,55]. A recent study found that *E. coli* emerges as the predominant bacteria involved in small intestine bacterial overgrowth and that NAFLD may be related to the efficient translocation abilities of these patients[56].

Hepatocyte triacylglycerol (TG) deposition is mainly due to three factors: lipolysis of adipose tissue, *de novo* lipogenesis, and TG dietary input, with contributions of 59%, 26%, and 15%, respectively. The excessive load of free fatty acid in the liver is the crucial cause of liver steatosis[57].

Dietary factors: Fructose and sugar
Carbohydrates can be converted to TG, and fructose is more closely associated with NAFLD compared to glucose. Fructose consumption, largely in the form of high fructose corn syrup (HFCS), a mixture of fructose and glucose monosaccharides, has increased over the past several decades[58]. Recent data suggest that diets high in sugar (sucrose and/or HFCS) not only increase the risk of NAFLD, but also of NASH. Indeed, fructose intake from added sugars in processed foods correlates with the epidemic rise in obesity, metabolic syndrome, and NAFLD. Fructose induced-hepatic fat accumulation involves the stress pathway that results in gluconeogenesis, an increase in fat synthesis, and a decrease in fat oxidation[59-61]. Fructose may modulate the lipogenic enzymes by increasing the expression of sterol regulatory element binding protein-1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP)[62]. Animal experiments[63] showed

that mice exposed to fructose with significant intestinal bacteria growth and increased intestinal permeability, as mentioned above, may trigger inflammation by increasing serum TNF-α.

Chronic fructose consumption induces leptin resistance prior to body weight, which accelerates high-fat induced obesity. Moreover, removal of fructose from this diet reverses leptin resistance and leptin augmentation, favoring a causal relationship[64,65].

Therefore, GLA is involved in the pathogenesis of NALFD, and GM dysbiosis promotes steatosis evolution to NASH. Special bacterial strains translocate more efficiently into the liver portal system. Further multicenter studies are required to test the bacterial genes of the normal population versus obese populations with IR (with and without NAFLD) with the aim of screening high-risk populations. In addition, improving intestinal dysbiosis and determining whether this improvement reduces the risk of NAFLD needs further investigation. These studies may pave the way for improving NAFLD diagnosis and treatment.

Genetic factors

Genetic factors are also important in the development of NAFLD. A certain genetic background has been shown to predispose an individual to fatty liver[66,67]. Those genes are involved in inflammation, lipid metabolism, and oxidation, and are associated with progressive liver disease, IR, type 2 diabetes mellitus, and a higher risk for hepatocellular carcinoma.

PNPLA3: *PNPLA3* is the most documented NAFLD-related gene. A genome-wide association study (GWAS) showed that the hepatic fat content of *PNPLA3* I148M allele carriers was more than 2-fold higher than in non-carriers, and a new variant *PNPLA3* S453I allele was identified which was associated with a significantly lower liver fat content particularly in African Americans[68]. Several studies have shown that *PNPLA3* I148M increases the risk of NAFLD without a strong effect on metabolic syndrome (MS) components, but abdominal fat (which is closely correlated to MS components) can drive the effect of this polymorphism on liver damage[69,70]. In obese children, weight loss can weaken the effect of this polymorphism[71].

McGeoch *et al*[72] suggested that patients with *PNPLA3* p.I148M showed the greatest response to the fructose-restricted diet, whereas those lacking this variant exhibit minimal or no change from baseline. Wang *et al*[73] revealed that physical activity and sedentary behavior can modulate the effect of the *PNPLA3* variant on childhood NAFLD. These evidences provide new clues to the function of the *PNPLA3* gene and are also useful for risk assessment and personalized treatment of NAFLD in the future.

Glucokinase regulator protein: Glucokinase regulator protein (GCKR) is an inhibitor of glucokinase

(GCK). GCK regulates glucose storage and disposal in the liver where its activity is regulated by GCKR. The GCKR genotype has been shown to modulate lipogenesis and fibrosis progression in NAFLD[74]. The combined effects of PNPLA3 rs738409 and GCKR rs1260326 polymorphisms account for up to one-third of variability in liver fat content in obese children[75,76].

Apolipoprotein C-III: Apolipoprotein C-III (*APOC3*) can inhibit the lipoprotein lipase and reduce the clearance of TG. In NAFLD, *APOC3* variants may lead to higher plasma concentrations of apolipoprotein C3 ending up in lower clearance. The consequence of the reduced TG clearance is an increase in residual particles of chylomicrons, that will lead to higher levels of circulating chylomicron remnants, which are especially cleared by the liver through a receptor-mediated process[77,78]. However, a recent study of *APOC3* transgenic mice suggested that *APOC3* dysregulation is not a predisposing factor for linking over-nutrition to NAFLD in obesity[79].

TM6SF2: Transmembrane 6 superfamily 2 (*TM6SF2*) has been recognized to regulate plasma lipids. On the basis of sequence similarity to Emopamil-binding protein (an enzyme with sterol isomerase activity), *TM6SF2* has been hypothesized to play a role in sterol biosynthesis[80,81]. Smagris *et al*[82] reported that *TM6SF2* is involved in the transfer of neutral lipids from cytoplasmic to luminal lipid droplets or very low density lipoprotein (VLDL) particles. Recently, variants of *TM6SF2* have been found to influence metabolic traits through alteration of protein stability[83-86].

PPARGC1A: Peroxisome proliferator activated receptor γ coactivator 1α (PGC-1α), expressing the *PPARGC1A* gene, is involved in the key steps of NAFLD development, such as insulin resistance, mitochondrial biogenesis, and oxidative phosphorylation[87,88]. In hepatocytes, PGC-1a orchestrates broad energy programs, including gluconeogenesis and mitochondrial fatty acid β-oxidation[89]. Moreover, *PPARGC1A* has been shown to regulate several key genes in hepatic gluconeogenesis (*CREB*, *PPARα*, *FOXO1*, *TRB-3*)[90-93]. *PPARGC1A* knockout mice reportedly developed hepatic steatosis due to a combination of reduced mitochondrial respiratory capacity and increased the expression of lipogenic genes[94].

Human microsomal triglyceride transfer protein: The human microsomal triglyceride transfer protein (MTTP) is involved in lipid transfer function and is critical for the assembly and secretion of VLDL to remove lipids from the liver. Thus, genetic polymorphisms in the *MTTP* gene may contribute to altered lipid metabolism by disrupting the assembly and secretion of lipoproteins, leading to reduced fat export from the involved hepatocytes and to NAFLD. Several genetic

polymorphisms in the *MTTP* gene have been identified; some are related to the pathogenesis of NAFLD while others interact with age, insulin resistance, and BMI and increase the risk for NAFLD[95-99].

Other genes: Recently, Buch *et al*[100] and Umano *et al*[101] identified the rs626283 variants in the *MBOAT7* gene as risk loci for alcohol-related cirrhosis in adults and obese youth[100,101]. In the Japanese population, the *SAMM50* gene (rs738491, rs3761472, and rs2143571), *PARVB* gene (rs6006473, rs5764455 and rs6006611), and *GATAD2A* gene (rs4808199) were found to be significantly associated with NAFLD[102,103].

Meanwhile, Chinese children with NAFLD presented a higher prevalence of *UCP3* gene rs11235972 GG[104]. Adams *et al*[105] reported that SNPs in two hepatic genes were associated with NAFLD in adolescents: The group-specific component and the lymphocyte cytosolic protein-1.

CONCLUSION

The pathogenesis of NAFLD and its progression is a complex process, in which some questions remain unanswered. The initial "two-hit" theory can no longer completely explain the pathogenesis of NAFLD, which involves multiple factors. In recent decades, many experiments have suggested that the gut microbiome plays a key role in NAFLD pathogenesis *via* the GLA. More recently, with the development of technology (especially GWAS), increasing studies have focused on genetic predispositions and found various gene variants that may alter lipid and sugar metabolism in the liver as well as in other tissues such as adipose tissue. Given the multifactorial nature of the related diseases, it may not be possible to obtain a single indicator that could precisely differentiate NAFLD and NASH. However, data in the future could be more promising in terms of population screening, with the goal to identify individuals at risk for NAFLD.

Hopefully, the "multiple hit model" (once further refined) will pave the way for tailoring therapeutics to genetic predispositions to NAFLD and NASH.

ACKNOWLEDGMENTS

REFERENCES

1   **Angulo P**. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221-1231 [PMID: 11961152 DOI: 10.1056/NEJMra011775]

2   **Tiniakos DG**, Vos MB, Brunt EM. Nonalcoholic fatty liver disease: pathology and pathogenesis. *Annu Rev Pathol* 2010; **5**: 145-171 [PMID: 20078219 DOI: 10.1146/annurev-pathol-121808-102132]

3   **Vernon G**, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Aliment Pharmacol Ther* 2011; **34**: 274-285 [PMID: 21623852 DOI: 10.1111/j.1365-2036.2011.04724.x]

4   **Farrell GC**, Wong VW, Chitturi S. NAFLD in Asia--as common and important as in the West. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 307-318 [PMID: 23458891 DOI: 10.1038/nrgastro.2013.34]

5   **Younossi ZM**, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 2016; **64**: 73-84 [PMID: 26707365 DOI: 10.1002/hep.28431]

6   **Schwimmer JB**, Deutsch R, Kahen T, Lavine JE, Stanley C, Behling C. Prevalence of fatty liver in children and adolescents. *Pediatrics* 2006; **118**: 1388-1393 [PMID: 17015527 DOI: 10.1542/peds.2006-1212]

7   **Barlow SE**; Expert Committee. Expert committee recommendations regarding the prevention, assessment, and treatment of child and adolescent overweight and obesity: summary report. *Pediatrics* 2007; **120** Suppl 4: S164-S192 [PMID: 18055651 DOI: 10.1542/peds.2007-2329C]

8   **Vajro P**, Lenta S, Socha P, Dhawan A, McKiernan P, Baumann U, Durmaz O, Lacaille F, McLin V, Nobili V. Diagnosis of nonalcoholic fatty liver disease in children and adolescents: position paper of the ESPGHAN Hepatology Committee. *J Pediatr Gastroenterol Nutr* 2012; **54**: 700-713 [PMID: 22395188 DOI: 10.1097/MPG.0b013e318252a13f]

9   **Vos MB**, Abrams SH, Barlow SE, Caprio S, Daniels SR, Kohli R, Mouzaki M, Sathya P, Schwimmer JB, Sundaram SS, Xanthakos SA. NASPGHAN Clinical Practice Guideline for the Diagnosis and Treatment of Nonalcoholic Fatty Liver Disease in Children: Recommendations from the Expert Committee on NAFLD (ECON) and the North American Society of Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN). *J Pediatr Gastroenterol Nutr* 2017; **64**: 319-334 [PMID: 28107283 DOI: 10.1097/MPG.0000000000001482]

10 **Zou CC**, Liang L, Hong F, Fu JF, Zhao ZY. Serum adiponectin, resistin levels and non-alcoholic fatty liver disease in obese children. *Endocr J* 2005; **52**: 519-524 [PMID: 16284427 DOI: 10.1507/endocrj.52.519]

11 **Zou CC**, Liang L, Zhao ZY. Factors associated with fasting plasma ghrelin levels in children and adolescents. *World J Gastroenterol* 2008; **14**: 790-794 [PMID: 18205273 DOI: 10.3748/wjg.14.790]

12 **Wang XM**, Jiang YJ, Liang L, Du LZ. Changes of ghrelin following oral glucose tolerance test in obese children with insulin resistance. *World J Gastroenterol* 2008; **14**: 1919-1924 [PMID: 18350633 DOI: 10.3748/wjg.14.1919]

13 **Fu JF**, Shi HB, Liu LR, Jiang P, Liang L, Wang CL, Liu XY. Non-alcoholic fatty liver disease: An early mediator predicting metabolic syndrome in obese children? *World J Gastroenterol* 2011; **17**: 735-742 [PMID: 21390143 DOI: 10.3748/wjg.v17.i6.735]

14 **Chen LH**, Liang L, Fang YL, Wang YM, Zhu WF. Fish oil improves lipid profile in juvenile rats with intrauterine growth retardation by altering the transcriptional expression of lipid-related hepatic genes. *J Matern Fetal Neonatal Med* 2016; **29**: 3292-3298 [PMID: 26586306 DOI: 10.3109/14767058.2015.1123244]

15 **Welsh JA**, Karpen S, Vos MB. Increasing prevalence of nonalcoholic fatty liver disease among United States adolescents, 1988-1994 to 2007-2010. *J Pediatr* 2013; **162**: 496-500.e1 [PMID: 23084707 DOI: 10.1016/j.jpeds.2012.08.043]

16 **Marzuillo P**, Miraglia del Giudice E, Santoro N. Pediatric fatty liver disease: role of ethnicity and genetics. *World J Gastroenterol* 2014; **20**: 7347-7355 [PMID: 24966605 DOI: 10.3748/wjg.v20.i23.7347]

17 **Giorgio V**, Prono F, Graziano F, Nobili V. Pediatric non alcoholic fatty liver disease: old and new concepts on development, progression, metabolic insight and potential treatment targets. *BMC Pediatr* 2013; **13**: 40 [PMID: 23530957 DOI: 10.1186/1471-2431-13-40]

18 **Schwimmer JB**, McGreal N, Deutsch R, Finegold MJ, Lavine JE. Influence of gender, race, and ethnicity on suspected fatty liver in obese adolescents. *Pediatrics* 2005; **115**: e561-e565 [PMID:

15867021 DOI: 10.1542/peds.2004-1832]

19   **Conjeevaram Selvakumar PK**, Kabbany MN, Alkhouri N. Nonalcoholic Fatty Liver Disease in Children: Not a Small Matter. *Paediatr Drugs* 2018; Epub ahead of print [PMID: 29740791 DOI: 10.1007/s40272-018-0292-2]

20   **Dowman JK**, Tomlinson JW, Newsome PN. Pathogenesis of non-alcoholic fatty liver disease. *QJM* 2010; **103**: 71-83 [PMID: 19914930 DOI: 10.1093/qjmed/hcp158]

21   **Schwimmer JB**, Behling C, Newbury R, Deutsch R, Nievergelt C, Schork NJ, Lavine JE. Histopathology of pediatric nonalcoholic fatty liver disease. *Hepatology* 2005; **42**: 641-649 [PMID: 16116629 DOI: 10.1002/hep.20842]

22   **Wang CL**, Liang L, Fu JF, Zou CC, Hong F, Xue JZ, Lu JR, Wu XM. Effect of lifestyle intervention on non-alcoholic fatty liver disease in Chinese obese children. *World J Gastroenterol* 2008; **14**: 1598-1602 [PMID: 18330955 DOI: 10.3748/wjg.14.1598]

23   **Fu JF**, Fang YL, Liang L, Wang CL, Hong F, Dong GP. A rabbit model of pediatric nonalcoholic steatohepatitis: the role of adiponectin. *World J Gastroenterol* 2009; **15**: 912-918 [PMID: 19248189 DOI: 10.3748/wjg.15.912]

24   **Ratziu V**, Bellentani S, Cortez-Pinto H, Day C, Marchesini G. A position statement on NAFLD/NASH based on the EASL 2009 special conference. *J Hepatol* 2010; **53**: 372-384 [PMID: 20494470 DOI: 10.1016/j.jhep.2010.04.008]

25   **Berardis S**, Sokal E. Pediatric non-alcoholic fatty liver disease: an increasing public health issue. *Eur J Pediatr* 2014; **173**: 131-139 [PMID: 24068459 DOI: 10.1007/s00431-013-2157-6]

26   **Alisi A**, Cianfarani S, Manco M, Agostoni C, Nobili V. Non-alcoholic fatty liver disease and metabolic syndrome in adolescents: pathogenetic role of genetic background and intrauterine environment. *Ann Med* 2012; **44**: 29-40 [PMID: 21355790 DOI: 10.3109/07853890.2010.547869]

27   **Ayonrinde OT**, Olynyk JK, Marsh JA, Beilin LJ, Mori TA, Oddy WH, Adams LA. Childhood adiposity trajectories and risk of nonalcoholic fatty liver disease in adolescents. *J Gastroenterol Hepatol* 2015; **30**: 163-171 [PMID: 24989077 DOI: 10.1111/jgh.12666]

28   **Musso G**, Gambino R, Cassader M. Cholesterol metabolism and the pathogenesis of non-alcoholic steatohepatitis. *Prog Lipid Res* 2013; **52**: 175-191 [PMID: 23206728 DOI: 10.1016/j.plipres.2012.11.002]

29   **Ferramosca A**, Zara V. Modulation of hepatic steatosis by dietary fatty acids. *World J Gastroenterol* 2014; **20**: 1746-1755 [PMID: 24587652 DOI: 10.3748/wjg.v20.i7.1746]

30   **Buzzetti E**, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Metabolism* 2016; **65**: 1038-1048 [PMID: 26823198 DOI: 10.1016/j.metabol.2015.12.012]

31   **Jacome-Sosa MM**, Parks EJ. Fatty acid sources and their fluxes as they contribute to plasma triglyceride concentrations and fatty liver in humans. *Curr Opin Lipidol* 2014; **25**: 213-220 [PMID: 24785962 DOI: 10.1097/MOL.0000000000000080]

32   **Rebrin K**, Steil GM, Mittelman SD, Bergman RN. Causal linkage between insulin suppression of lipolysis and suppression of liver glucose output in dogs. *J Clin Invest* 1996; **98**: 741-749 [PMID: 8698866 DOI: 10.1172/JCI118846]

33   **Petersen MC**, Shulman GI. Roles of Diacylglycerols and Ceramides in Hepatic Insulin Resistance. *Trends Pharmacol Sci* 2017; **38**: 649-665 [PMID: 28551355 DOI: 10.1016/j.tips.2017.04.004]

34   **Shulman GI**. Ectopic fat in insulin resistance, dyslipidemia, and cardiometabolic disease. *N Engl J Med* 2014; **371**: 2237-2238 [PMID: 25470706 DOI: 10.1056/NEJMc1412427]

35   **Hotamisligil GS**. Inflammation and metabolic disorders. *Nature* 2006; **444**: 860-867 [PMID: 17167474 DOI: 10.1038/nature05485]

36   **Cai D**, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J, Shoelson SE. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med* 2005; **11**: 183-190 [PMID: 15685173 DOI: 10.1038/nm1166]

37   **Ribeiro PS**, Cortez-Pinto H, Solá S, Castro RE, Ramalho RM, Baptista A, Moura MC, Camilo ME, Rodrigues CM. Hepatocyte apoptosis, expression of death receptors, and activation of NF-kappaB in the liver of nonalcoholic and alcoholic steatohepatitis patients. *Am J Gastroenterol* 2004; **99**: 1708-1717 [PMID: 15330907 DOI: 10.1111/j.1572-0241.2004.40009.x]

38   **Tomita K**, Tamiya G, Ando S, Ohsumi K, Chiyo T, Mizutani A, Kitamura N, Toda K, Kaneko T, Horie Y, Han JY, Kato S, Shimoda M, Oike Y, Tomizawa M, Makino S, Ohkura T, Saito H, Kumagai N, Nagata H, Ishii H, Hibi T. Tumour necrosis factor alpha signalling through activation of Kupffer cells plays an essential role in liver fibrosis of non-alcoholic steatohepatitis in mice. *Gut* 2006; **55**: 415-424 [PMID: 16174657 DOI: 10.1136/gut.2005.071118]

39   **Thomson AW**, Knolle PA. Antigen-presenting cell function in the tolerogenic liver environment. *Nat Rev Immunol* 2010; **10**: 753-766 [PMID: 20972472 DOI: 10.1038/nri2858]

40   **Lotze MT**, Zeh HJ, Rubartelli A, Sparvero LJ, Amoscato AA, Washburn NR, Devera ME, Liang X, Tör M, Billiar T. The grateful dead: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity. *Immunol Rev* 2007; **220**: 60-81 [PMID: 17979840 DOI: 10.1111/j.1600-065X.2007.00579.x]

41   **Szabo G**, Csak T. Inflammasomes in liver diseases. *J Hepatol* 2012; **57**: 642-654 [PMID: 22634126 DOI: 10.1016/j.jhep.2012.03.035]

42   **Luedde T**, Schwabe RF. NF-κB in the liver--linking injury, fibrosis and hepatocellular carcinoma. *Nat Rev Gastroenterol Hepatol* 2011; **8**: 108-118 [PMID: 21293511 DOI: 10.1038/nrgastro.2010.213]

43   **Klein I**, Cornejo JC, Polakos NK, John B, Wuensch SA, Topham DJ, Pierce RH, Crispe IN. Kupffer cell heterogeneity: functional properties of bone marrow derived and sessile hepatic macrophages. *Blood* 2007; **110**: 4077-4085 [PMID: 17690256 DOI: 10.1182/blood-2007-02-073841]

44   **Miura K**, Kodama Y, Inokuchi S, Schnabl B, Aoyama T, Ohnishi H, Olefsky JM, Brenner DA, Seki E. Toll-like receptor 9 promotes steatohepatitis by induction of interleukin-1beta in mice. *Gastroenterology* 2010; **139**: 323-334.e7 [PMID: 20347818 DOI: 10.1053/j.gastro.2010.03.052]

45   **Miura K**, Yang L, van Rooijen N, Brenner DA, Ohnishi H, Seki E. Toll-like receptor 2 and palmitic acid cooperatively contribute to the development of nonalcoholic steatohepatitis through inflammasome activation in mice. *Hepatology* 2013; **57**: 577-589 [PMID: 22987396 DOI: 10.1002/hep.26081]

46   **Clemente MG**, Mandato C, Poeta M, Vajro P. Pediatric non-alcoholic fatty liver disease: Recent solutions, unresolved issues, and future research directions. *World J Gastroenterol* 2016; **22**: 8078-8093 [PMID: 27688650 DOI: 10.3748/wjg.v22.i36.8078]

47   **Rotman Y**, Sanyal AJ. Current and upcoming pharmacotherapy for non-alcoholic fatty liver disease. *Gut* 2017; **66**: 180-190 [PMID: 27646933 DOI: 10.1136/gutjnl-2016-312431]

48   **Poeta M**, Pierri L, Vajro P. Gut-Liver Axis Derangement in Non-Alcoholic Fatty Liver Disease. *Children* (Basel) 2017; **4**: 66 [PMID: 28767077 DOI: 10.3390/children4080066]

49   **Paolella G**, Mandato C, Pierri L, Poeta M, Di Stasi M, Vajro P. Gut-liver axis and probiotics: their role in non-alcoholic fatty liver disease. *World J Gastroenterol* 2014; **20**: 15518-15531 [PMID: 25400436 DOI: 10.3748/wjg.v20.i42.15518]

50   **Zorn AM**, Wells JM. Vertebrate endoderm development and organ formation. *Annu Rev Cell Dev Biol* 2009; **25**: 221-251 [PMID: 19575677 DOI: 10.1146/annurev.cellbio.042308.113344]

51   **Lozupone CA**, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. *Nature* 2012; **489**: 220-230 [PMID: 22972295 DOI: 10.1038/nature11550]

52   **Doulberis M**, Kotronis G, Gialamprinou D, Kountouras J, Katsinelos P. Non-alcoholic fatty liver disease: An update with special focus on the role of gut microbiota. *Metabolism* 2017; **71**: 182-197 [PMID: 28521872 DOI: 10.1016/j.metabol.2017.03.013]

53   **Ulluwishewa D**, Anderson RC, McNabb WC, Moughan PJ, Wells JM, Roy NC. Regulation of tight junction permeability by intestinal bacteria and dietary components. *J Nutr* 2011; **141**: 769-776 [PMID: 21430248 DOI: 10.3945/jn.110.135657]

54   **Rahman K**, Desai C, Iyer SS, Thorn NE, Kumar P, Liu Y, Smith T, Neish AS, Li H, Tan S, Wu P, Liu X, Yu Y, Farris AB, Nusrat A, Parkos CA, Anania FA. Loss of Junctional Adhesion Molecule A Promotes Severe Steatohepatitis in Mice on a Diet High in Saturated Fat, Fructose, and Cholesterol. *Gastroenterology* 2016; **151**: 733-746.e12 [PMID: 27342212 DOI: 10.1053/j.gastro.2016.06.022]

55   **Miele L**, Valenza V, La Torre G, Montalto M, Cammarota G, Ricci R, Mascianà R, Forgione A, Gabrieli ML, Perotti G, Vecchio FM, Rapaccini G, Gasbarrini G, Day CP, Grieco A. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. *Hepatology* 2009; **49**: 1877-1887 [PMID: 19291785 DOI: 10.1002/hep.22848]

56   **Kapil S**, Duseja A, Sharma BK, Singla B, Chakraborti A, Das A, Ray P, Dhiman RK, Chawla Y. Small intestinal bacterial overgrowth and toll-like receptor signaling in patients with non-alcoholic fatty liver disease. *J Gastroenterol Hepatol* 2016; **31**: 213-221 [PMID: 26212089 DOI: 10.1111/jgh.13058]

57   **Donnelly KL**, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest* 2005; **115**: 1343-1351 [PMID: 15864352 DOI: 10.1172/JCI23621]

58   **Ventura EE**, Davis JN, Goran MI. Sugar content of popular sweetened beverages based on objective laboratory analysis: focus on fructose content. *Obesity* (Silver Spring) 2011; **19**: 868-874 [PMID: 20948525 DOI: 10.1038/oby.2010.255]

59   **Lanaspa MA**, Sanchez-Lozada LG, Choi YJ, Cicerchi C, Kanbay M, Roncal-Jimenez CA, Ishimoto T, Li N, Marek G, Duranay M, Schreiner G, Rodriguez-Iturbe B, Nakagawa T, Kang DH, Sautin YY, Johnson RJ. Uric acid induces hepatic steatosis by generation of mitochondrial oxidative stress: potential role in fructose-dependent and -independent fatty liver. *J Biol Chem* 2012; **287**: 40732-40744 [PMID: 23035112 DOI: 10.1074/jbc.M112.399899]

60   **Lanaspa MA**, Cicerchi C, Garcia G, Li N, Roncal-Jimenez CA, Rivard CJ, Hunter B, Andrés-Hernando A, Ishimoto T, Sánchez-Lozada LG, Thomas J, Hodges RS, Mant CT, Johnson RJ. Counteracting roles of AMP deaminase and AMP kinase in the development of fatty liver. *PLoS One* 2012; **7**: e48801 [PMID: 23152807 DOI: 10.1371/journal.pone.0048801]

61   **Cicerchi C**, Li N, Kratzer J, Garcia G, Roncal-Jimenez CA, Tanabe K, Hunter B, Rivard CJ, Sautin YY, Gaucher EA, Johnson RJ, Lanaspa MA. Uric acid-dependent inhibition of AMP kinase induces hepatic glucose production in diabetes and starvation: evolutionary implications of the uricase loss in hominids. *FASEB J* 2014; **28**: 3339-3350 [PMID: 24755741 DOI: 10.1096/fj.13-243634]

62   **Postic C**, Girard J. Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. *J Clin Invest* 2008; **118**: 829-838 [PMID: 18317565 DOI: 10.1172/JCI34275]

63   **Spruss A**, Kanuri G, Wagnerberger S, Haub S, Bischoff SC, Bergheim I. Toll-like receptor 4 is involved in the development of fructose-induced hepatic steatosis in mice. *Hepatology* 2009; **50**: 1094-1104 [PMID: 19637282 DOI: 10.1002/hep.23122]

64   **Shapiro A**, Mu W, Roncal C, Cheng KY, Johnson RJ, Scarpace PJ. Fructose-induced leptin resistance exacerbates weight gain in response to subsequent high-fat feeding. *Am J Physiol Regul Integr Comp Physiol* 2008; **295**: R1370-R1375 [PMID: 18703413 DOI: 10.1152/ajpregu.00195.2008]

65   **Shapiro A**, Tümer N, Gao Y, Cheng KY, Scarpace PJ. Prevention and reversal of diet-induced leptin resistance with a sugar-free diet despite high fat content. *Br J Nutr* 2011; **106**: 390-397 [PMID: 21418711 DOI: 10.1017/S000711451100033X]

66   **Browning JD**, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, Hobbs HH. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 2004; **40**: 1387-1395 [PMID: 15565570 DOI: 10.1002/hep.20466]

67   **Loomba R**, Sanyal AJ. The global NAFLD epidemic. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 686-690 [PMID: 24042449 DOI: 10.1038/nrgastro.2013.171]

68   **Romeo S**, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, Boerwinkle E, Cohen JC, Hobbs HH. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. *Nat Genet* 2008; **40**: 1461-1465 [PMID: 18820647 DOI: 10.1038/ng.257]

69   **Giudice EM**, Grandone A, Cirillo G, Santoro N, Amato A, Brienza C, Savarese P, Marzuillo P, Perrone L. The association of PNPLA3 variants with liver enzymes in childhood obesity is driven by the interaction with abdominal fat. *PLoS One* 2011; **6**: e27933 [PMID: 22140488 DOI: 10.1371/journal.pone.0027933]

70   **Speliotes EK**, Butler JL, Palmer CD, Voight BF; GIANT Consortium; MIGen Consortium; NASH CRN, Hirschhorn JN. PNPLA3 variants specifically confer increased risk for histologic nonalcoholic fatty liver disease but not metabolic disease. *Hepatology* 2010; **52**: 904-912 [PMID: 20648472 DOI: 10.1002/hep.23768]

71   **Marzuillo P**, Grandone A, Perrone L, del Giudice EM. Weight loss allows the dissection of the interaction between abdominal fat and PNPLA3 (adiponutrin) in the liver damage of obese children. *J Hepatol* 2013; **59**: 1143-1144 [PMID: 23845393 DOI: 10.1016/j.jhep.2013.06.027]

72   **McGeoch LJ**, Patel PR, Mann JP. PNPLA3: A Determinant of Response to Low-Fructose Diet in Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2018; **154**: 1207-1208 [PMID: 29452087 DOI: 10.1053/j.gastro.2017.07.054]

73   **Wang S**, Song J, Shang X, Chawla N, Yang Y, Meng X, Wang H, Ma J. Physical activity and sedentary behavior can modulate the effect of the PNPLA3 variant on childhood NAFLD: a case-control study in a Chinese population. *BMC Med Genet* 2016; **17**: 90 [PMID: 27905898 DOI: 10.1186/s12881-016-0352-9]

74   **Petta S**, Miele L, Bugianesi E, Cammà C, Rosso C, Boccia S, Cabibi D, Di Marco V, Grimaudo S, Grieco A, Pipitone RM, Marchesini G, Craxì A. Glucokinase regulatory protein gene polymorphism affects liver fibrosis in non-alcoholic fatty liver disease. *PLoS One* 2014; **9**: e87523 [PMID: 24498332 DOI: 10.1371/journal.pone.0087523]

75   **Santoro N**, Zhang CK, Zhao H, Pakstis AJ, Kim G, Kursawe R, Dykas DJ, Bale AE, Giannini C, Pierpont B, Shaw MM, Groop L, Caprio S. Variant in the glucokinase regulatory protein (GCKR) gene is associated with fatty liver in obese children and adolescents. *Hepatology* 2012; **55**: 781-789 [PMID: 22105854 DOI: 10.1002/hep.24806]

76   **Valenti L**, Alisi A, Nobili V. Unraveling the genetics of fatty liver in obese children: additive effect of P446L GCKR and I148M PNPLA3 polymorphisms. *Hepatology* 2012; **55**: 661-663 [PMID: 22281838 DOI: 10.1002/hep.25617]

77   **Windler EE**, Greeve J, Daerr WH, Greten H. Binding of rat chylomicrons and their remnants to the hepatic low-density-lipoprotein receptor and its role in remnant removal. *Biochem J* 1988; **252**: 553-561 [PMID: 3415673 DOI: 10.1042/bj2520553]

78   **Nagata Y**, Chen J, Cooper AD. Role of low density lipoprotein receptor-dependent and -independent sites in binding and uptake of chylomicron remnants in rat liver. *J Biol Chem* 1988; **263**: 15151-15158 [PMID: 3170577]

79   **Cheng X**, Yamauchi J, Lee S, Zhang T, Gong Z, Muzumdar R, Qu S, Dong HH. APOC3 Protein Is Not a Predisposing Factor for Fat-induced Nonalcoholic Fatty Liver Disease in Mice. *J Biol Chem* 2017; **292**: 3692-3705 [PMID: 28115523 DOI: 10.1074/jbc.M116.765917]

80   **Fan Y**, Lu H, Guo Y, Zhu T, Garcia-Barrio MT, Jiang Z, Willer CJ, Zhang J, Chen YE. Hepatic Transmembrane 6 Superfamily Member 2 Regulates Cholesterol Metabolism in Mice. *Gastroenterology* 2016; **150**: 1208-1218 [PMID: 26774178 DOI: 10.1053/j.gastro.2016.01.005]

81   **Sanchez-Pulido L**, Ponting CP. TM6SF2 and MAC30, new enzyme homologs in sterol metabolism and common metabolic disease. *Front Genet* 2014; **5**: 439 [PMID: 25566323 DOI: 10.3389/fgene.2014.00439]

82   **Smagris E**, Gilyard S, BasuRay S, Cohen JC, Hobbs HH. Inactivation of Tm6sf2, a Gene Defective in Fatty Liver Disease, Impairs Lipidation but Not Secretion of Very Low Density Lipoproteins. *J Biol Chem* 2016; **291**: 10659-10676 [PMID: 27013658 DOI: 10.1074/jbc.M116.719955]

83   **Holmen OL**, Zhang H, Fan Y, Hovelson DH, Schmidt EM, Zhou W, Guo Y, Zhang J, Langhammer A, Løchen ML, Ganesh SK, Vatten L, Skorpen F, Dalen H, Zhang J, Pennathur S, Chen J, Platou C, Mathiesen EB, Wilsgaard T, Njølstad I, Boehnke M, Chen YE, Abecasis GR, Hveem K, Willer CJ. Systematic evaluation of coding variation identifies a candidate causal variant in TM6SF2 influencing total cholesterol and myocardial infarction risk. *Nat Genet* 2014; **46**: 345-351 [PMID: 24633158 DOI: 10.1038/ng.2926]

84   **Kozlitina J**, Smagris E, Stender S, Nordestgaard BG, Zhou HH, Tybjærg-Hansen A, Vogt TF, Hobbs HH, Cohen JC. Exome-wide association study identifies a TM6SF2 variant that confers susceptibility to nonalcoholic fatty liver disease. *Nat Genet* 2014; **46**: 352-356 [PMID: 24531328 DOI: 10.1038/ng.2901]

85   **Surakka I**, Horikoshi M, Mägi R, Sarin AP, Mahajan A, Lagou V, Marullo L, Ferreira T, Miraglio B, Timonen S, Kettunen J, Pirinen M, Karjalainen J, Thorleifsson G, Hägg S, Hottenga JJ, Isaacs A, Ladenvall C, Beekman M, Esko T, Ried JS, Nelson CP, Willenborg C, Gustafsson S, Westra HJ, Blades M, de Craen AJ, de Geus EJ, Deelen J, Grallert H, Hamsten A, Havulinna AS, Hengstenberg C, Houwing-Duistermaat JJ, Hyppönen E, Karssen LC, Lehtimäki T, Lyssenko V, Magnusson PK, Mihailov E, Müller-Nurasyid M, Mpindi JP, Pedersen NL, Penninx BW, Perola M, Pers TH, Peters A, Rung J, Smit JH, Steinthorsdottir V, Tobin MD, Tsernikova N, van Leeuwen EM, Viikari JS, Willems SM, Willemsen G, Schunkert H, Erdmann J, Samani NJ, Kaprio J, Lind L, Gieger C, Metspalu A, Slagboom PE, Groop L, van Duijn CM, Eriksson JG, Jula A, Salomaa V, Boomsma DI, Power C, Raitakari OT, Ingelsson E, Järvelin MR, Thorsteinsdottir U, Franke L, Ikonen E, Kallioniemi O, Pietiäinen V, Lindgren CM, Stefansson K, Palotie A, McCarthy MI, Morris AP, Prokopenko I, Ripatti S; ENGAGE Consortium. The impact of low-frequency and rare variants on lipid levels. *Nat Genet* 2015; **47**: 589-597 [PMID: 25961943 DOI: 10.1038/ng.3300]

86   **Ehrhardt N**, Doche ME, Chen S, Mao HZ, Walsh MT, Bedoya C, Guindi M, Xiong W, Ignatius Irudayam J, Iqbal J, Fuchs S, French SW, Mahmood Hussain M, Arditi M, Arumugaswami V, Péterfy M. Hepatic Tm6sf2 overexpression affects cellular ApoB-trafficking, plasma lipid levels, hepatic steatosis and atherosclerosis. *Hum Mol Genet* 2017; **26**: 2719-2731 [PMID: 28449094 DOI: 10.1093/hmg/ddx159]

87   **Lin YC**, Chang PF, Chang MH, Ni YH. A common variant in the peroxisome proliferator-activated receptor-γ coactivator-1α gene is associated with nonalcoholic fatty liver disease in obese children. *Am J Clin Nutr* 2013; **97**: 326-331 [PMID: 23269818 DOI: 10.3945/ajcn.112.046417]

88   **Sookoian S**, Rosselli MS, Gemma C, Burgueño AL, Fernández Gianotti T, Castaño GO, Pirola CJ. Epigenetic regulation of insulin resistance in nonalcoholic fatty liver disease: impact of liver methylation of the peroxisome proliferator-activated receptor γ coactivator 1α promoter. *Hepatology* 2010; **52**: 1992-2000 [PMID: 20890895 DOI: 10.1002/hep.23927]

89   **Lin J**, Handschin C, Spiegelman BM. Metabolic control through the PGC-1 family of transcription coactivators. *Cell Metab* 2005; **1**: 361-370 [PMID: 16054085 DOI: 10.1016/j.cmet.2005.05.004]

90   **Herzig S**, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, Rudolph D, Schutz G, Yoon C, Puigserver P, Spiegelman B, Montminy M. CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature* 2001; **413**: 179-183 [PMID: 11557984 DOI: 10.1038/35093131]

91   **Yoon JC**, Puigserver P, Chen G, Donovan J, Wu Z, Rhee J, Adelmant G, Stafford J, Kahn CR, Granner DK, Newgard CB, Spiegelman BM. Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature* 2001; **413**: 131-138 [PMID: 11557972 DOI: 10.1038/35093050]

92   **Puigserver P**, Rhee J, Donovan J, Walkey CJ, Yoon JC, Oriente F, Kitamura Y, Altomonte J, Dong H, Accili D, Spiegelman BM. Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. *Nature* 2003; **423**: 550-555 [PMID: 12754525 DOI: 10.1038/nature01667]

93   **Koo SH**, Satoh H, Herzig S, Lee CH, Hedrick S, Kulkarni R, Evans RM, Olefsky J, Montminy M. PGC-1 promotes insulin resistance in liver through PPAR-alpha-dependent induction of TRB-3. *Nat Med* 2004; **10**: 530-534 [PMID: 15107844 DOI: 10.1038/nm1044]

94   **Leone TC**, Lehman JJ, Finck BN, Schaeffer PJ, Wende AR, Boudina S, Courtois M, Wozniak DF, Sambandam N, Bernal-Mizrachi C, Chen Z, Holloszy JO, Medeiros DM, Schmidt RE, Saffitz JE, Abel ED, Semenkovich CF, Kelly DP. PGC-1alpha deficiency causes multi-system energy metabolic derangements: muscle dysfunction, abnormal weight control and hepatic steatosis. *PLoS Biol* 2005; **3**: e101 [PMID: 15760270 DOI: 10.1371/journal.pbio.0030101]

95   **Dai D**, Wen F, Zhou S, Su Z, Liu G, Wang M, Zhou J, He F. Association of MTTP gene variants with pediatric NAFLD: A candidate-gene-based analysis of single nucleotide variations in obese children. *PLoS One* 2017; **12**: e0185396 [PMID: 28953935 DOI: 10.1371/journal.pone.0185396]

96   **Musso G**, Gambino R, Cassader M. Lipoprotein metabolism mediates the association of MTP polymorphism with beta-cell dysfunction in healthy subjects and in nondiabetic normolipidemic patients with nonalcoholic steatohepatitis. *J Nutr Biochem* 2010; **21**: 834-840 [PMID: 19733470 DOI: 10.1016/j.jnutbio.2009.06.007]

97   **Siqueira ER**, Oliveira CP, Correa-Giannella ML, Stefano JT, Cavaleiro AM, Fortes MA, Muniz MT, Silva FS, Pereira LM, Carrilho FJ. MTP -493G/T gene polymorphism is associated with steatosis in hepatitis C-infected patients. *Braz J Med Biol Res* 2012; **45**: 72-77 [PMID: 22147193 DOI: 10.1590/S0100-879X2011007500160]

98   **Peng XE**, Wu YL, Lu QQ, Hu ZJ, Lin X. MTTP polymorphisms and susceptibility to non-alcoholic fatty liver disease in a Han Chinese population. *Liver Int* 2014; **34**: 118-128 [PMID: 23738963 DOI: 10.1111/liv.12220]

99   **Hsiao PJ**, Lee MY, Wang YT, Jiang HJ, Lin PC, Yang YH, Kuo KK. MTTP-297H polymorphism reduced serum cholesterol but increased risk of non-alcoholic fatty liver disease-a cross-sectional study. *BMC Med Genet* 2015; **16**: 93 [PMID: 26458397 DOI: 10.1186/s12881-015-0242-6]

100   **Buch S**, Stickel F, Trépo E, Way M, Herrmann A, Nischalke HD, Brosch M, Rosendahl J, Berg T, Ridinger M, Rietschel M, McQuillin A, Frank J, Kiefer F, Schreiber S, Lieb W, Soyka M, Semmo N, Aigner E, Datz C, Schmelz R, Brückner S, Zeissig S, Stephan AM, Wodarz N, Devière J, Clumeck N, Sarrazin C, Lammert F, Gustot T, Deltenre P, Völzke H, Lerch MM, Mayerle J, Eyer F, Schafmayer C, Cichon S, Nöthen MM, Nothnagel M, Ellinghaus D, Huse K, Franke A, Zopf S, Hellerbrand C, Moreno C, Franchimont D, Morgan MY, Hampe J. A genome-wide association study confirms PNPLA3 and identifies TM6SF2 and MBOAT7 as risk loci for alcohol-related cirrhosis. *Nat Genet* 2015; **47**: 1443-1448 [PMID: 26482880 DOI: 10.1038/ng.3417]

101   **Umano GR**, Caprio S, Di Sessa A, Chalasani N, Dykas DJ, Pierpont B, Bale AE, Santoro N. The rs626283 Variant in the MBOAT7 Gene is Associated with Insulin Resistance and Fatty Liver in Caucasian Obese Youth. *Am J Gastroenterol* 2018; **113**: 376-383 [PMID: 29485130 DOI: 10.1038/ajg.2018.1]

102   **Kitamoto T**, Kitamoto A, Yoneda M, Hyogo H, Ochi H, Nakamura T, Teranishi H, Mizusawa S, Ueno T, Chayama K, Nakajima A, Nakao K, Sekine A, Hotta K. Genome-wide scan revealed that polymorphisms in the PNPLA3, SAMM50, and PARVB genes are associated with development and progression of nonalcoholic fatty liver disease in Japan. *Hum Genet* 2013; **132**: 783-792 [PMID: 23535911 DOI: 10.1007/s00439-013-1294-3]

103   **Kawaguchi T**, Shima T, Mizuno M, Mitsumoto Y, Umemura A, Kanbara Y, Tanaka S, Sumida Y, Yasui K, Takahashi M, Matsuo K,

Itoh Y, Tokushige K, Hashimoto E, Kiyosawa K, Kawaguchi M, Itoh H, Uto H, Komorizono Y, Shirabe K, Takami S, Takamura T, Kawanaka M, Yamada R, Matsuda F, Okanoue T. Risk estimation model for nonalcoholic fatty liver disease in the Japanese using multiple genetic markers. *PLoS One* 2018; **13**: e0185490 [PMID: 29385134 DOI: 10.1371/journal.pone.0185490]

104 **Xu YP**, Liang L, Wang CL, Fu JF, Liu PN, Lv LQ, Zhu YM. Association between UCP3 gene polymorphisms and nonalcoholic fatty liver disease in Chinese children. *World J Gastroenterol* 2013; **19**: 5897-5903 [PMID: 24124336 DOI: 10.3748/wjg.v19.i35.5897]

105 **Adams LA**, White SW, Marsh JA, Lye SJ, Connor KL, Maganga R, Ayonrinde OT, Olynyk JK, Mori TA, Beilin LJ, Palmer LJ, Hamdorf JM, Pennell CE. Association between liver-specific gene polymorphisms and their expression levels with nonalcoholic fatty liver disease. *Hepatology* 2013; **57**: 590-600 [PMID: 23213074 DOI: 10.1002/hep.26184]

Role of aldehyde dehydrogenase 2 in ischemia reperfusion injury: An update

Arnau Panisello-Roselló, Alexandre Lopez, Emma Folch-Puy, Teresa Carbonell, Anabela Rolo, Carlos Palmeira, René Adam, Marc Net, Joan Roselló-Catafau

Arnau Panisello-Roselló, Emma Folch-Puy, Joan Roselló-Catafau, Department of Experimental Pathology, Institute of Biomedical Research of Barcelona (IIBB)-CSIC, Barcelona 08036, Spain

Alexandre Lopez, René Adam, Centre Hepatobiliare, AP-HP Hôpital Paul Brousse, Villejuif 75008, France

Teresa Carbonell, Department of Physiology, Faculty of Biology, Universitat de Barcelona, Barcelona 08036, Spain

Anabela Rolo, Carlos Palmeira, Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra 3004-517, Portugal

Marc Net, Institute Georges Lopez, Lissieu 69380, France

ORCID number: Arnau Panisello-Roselló (0000-0003-2062 -6134); Alexandre Lopez (0000-0001-8978-4486); Emma Folch-Puy (0000-0002-6277-9027); Teresa Carbonell (0000-0002-7131 -3667); Anabela Rolo (0000-0003-3535-9630); Carlos Palmeira (0000-0002-2639-7697); Marc Net (0000-0003-0906-6404); Joan Roselló-Catafau (0000-0001-7127-4883).

Author contributions: All authors contributed equally to the writing of the manuscript and approved the final version.

Conflict-of-interest statement: The authors have no conflict of interests to declare.

Correspondence to: Joan Roselló-Catafau PhD, Adjunct Professor, Doctor, Department of Experimental Pathology, Institute of Biomedical Research of Barcelona (IIBB)-CSIC, C/ Roselló 161, Barcelona 08036, Spain. jrcbam@iibb.csic.es
Telephone: +34-93-3638333
Fax: +34-93-3638301

Abstract

Aldehyde dehydrogenase 2 (ALDH2) is best known for its critical detoxifying role in liver alcohol metabolism. However, ALDH2 dysfunction is also involved in a wide range of human pathophysiological situations and is associated with complications such as cardiovascular diseases, diabetes mellitus, neurodegenerative diseases and aging. A growing body of research has shown that ALDH2 provides important protection against oxidative stress and the subsequent loading of toxic aldehydes such as 4-hydroxy-2-nonenal and adducts that occur in human diseases, including ischemia reperfusion injury (IRI). There is increasing evidence of its role in IRI pathophysiology in organs such as heart, brain, small intestine and kidney; however, surprisingly few studies have been carried out in the liver, where ALDH2 is found in abundance. This study reviews the role of ALDH2 in modulating the pathways involved in the pathophysiology of IRI associated with oxidative stress, autophagy and apoptosis. Special emphasis is placed on the role of ALDH2 in different organs, on therapeutic "preconditioning" strategies, and on the use of ALDH2 agonists such as Alda-1, which may become a useful therapeutic tool for preventing the deleterious effects of IRI in organ transplantation.

Key words: Aldehyde dehydrogenase 2; 4-hydroxy-2-

nonenal autophagy; Apoptosis; Ischemia reperfusion injury; Preconditioning

Core tip: Aldehyde dehydrogenase 2 (ALDH2) plays a crucial role not only in liver ethanol metabolism but also in diverse pathophysiological dysfunctions including cardiovascular diseases, stroke, diabetes, neuro-degenerative dysfunctions, and aging. Its involvement has recently been identified in ischemia reperfusion injury (IRI). The present study provides an updated review of the literature on the role of ALDH2 in ischemia-reperfusion injury and its activation in different organs (heart, brain, kidney, intestine, *etc*) focusing especially on its possible use as a potential therapeutic target for preventing IRI associated with organ transplantation.

INTRODUCTION

Aldehyde dehydrogenase 2 (ALDH2), a tetrameric allosteric mitochondrial enzyme, is one of the 19 different ALDH enzymes ubiquitously expressed in all human tissues. It is most abundant in the liver, where it plays an essential role in the ethanol detoxifying pathway[1]. This pathway comprises two enzymatic steps. In the first, ethanol is metabolized by alcohol dehydrogenase (ADH) to form acetaldehyde, which is highly diffusible and crosses biological membranes, then circulates in the blood and is metabolized by ALDH2 to acetic acid[1,2]. ALDH2 is also responsible for the detoxification of other toxic short-term aldehydes, including some aromatic and polycyclic types[1,3]. In addition, ALDH2 oxidizes endogenous aldehydic products from lipid peroxidation of mitochondrial and plasma membranes under oxidative stress conditions, such as 4-hydroxy-2-nonenal (4-HNE) and lipoperoxides (malondialdehyde, MDA)[4,5]. The clearance of these harmful 4-HNE and aldehyde adducts, performed by ALDH2, is crucial for cell survival[1,4-6], since it is well known that 4-HNE affects mitochondrial and membrane integrity and other functions like apoptosis[7,8]. Although ALDH2 was initially known for its role in ethanol metabolism in liver[1], it has also been implicated in several pathologies, such as cardiovascular diseases[1,9], diabetes[1,10], neurologic dysfunctions[1,11] and more recently, in ischemia reperfusion injury (IRI) in organs such as heart[11-14], brain and eyes[15-19], intestine[20,21],

kidney[22,23] and spinal cord[24].

IRI, a process inherent in organ tumor resection and transplantation, affects organ quality and transplant outcomes. During the process, the organ deteriorates due to the lack of blood flow and oxygen deprivation (ischemic injury) and subsequently after the restoration of blood flow and oxygen supply (reperfusion injury). While some damage occurs during the ischemia phase, reperfusion by itself triggers a new set of detrimental cellular processes that provoke the energy metabolism breakdown exacerbating the injury and cell death. With the entry of oxygen to the organ, reactive oxygen species (ROS), lipoperoxides and toxic aldehydes are generated, inflammatory mediators are released and cell signaling pathways are activated, extending cell damage[25,26], exacerbating cell death processes, and eventually leading to organ failure.

In this review, we first present some general considerations on the role of ALDH2 in the complex pathophysiology of IRI/oxidative mechanisms, focusing on 4-HNE and its consequences for autophagy/apoptosis processes. Then we discuss the use of therapeutic strategies, such as "surgical preconditioning" (remote ischemic preconditioning and post-conditioning) and "pharmacological preconditioning" (isoflurane, ethanol; nitrite/nitrate strategies and ALDH2 agonists) for the prevention of IRI. In the final section, we offer an update on specific ALDH2 investigations carried out in different organs subjected to I/R such as heart, brain, eyes, intestine and kidney, and assess the possibility of using ALDH2 agonists to prevent cold IRI in the future, with special emphasis on liver transplantation.

ALDH2 AND 4-HNE

The mitochondria are the main source of the ROS generated during reperfusion. ROS interacts with polyunsaturated fatty acids in biological membranes, producing high toxic and reactive molecules such as 4-HNE[4,5], which can trigger the opening of mitochondrial permeability transition pores and inhibit the electron transport chain, thus contributing to the extension of the damage.

The specific electrophilic features of 4-HNE confer on this molecule a high reactivity and capacity to modify enzymes like kinases, belonging to sensitive pathways of cell homeostasis such as mitochondrial bioenergetics, redox balance, ROS formation, autophagy, apoptosis, and so on.

ALDH2 overexpression delays the formation of 4-HNE and toxic aldehyde adducts, and thus preserves the mitochondrial function during IRI[27,28]. Nevertheless, despite the ability of ALDH2 to remove these aldehyde adducts, when 4-HNE is accumulated at high concentrations, it may act as an ALDH2 inhibitor *in vitro*, countering its beneficial effects and worsening the impact of I/R insult[12,29]. 4-HNE has been shown to inflict organelle damage in a wide variety of cell components,
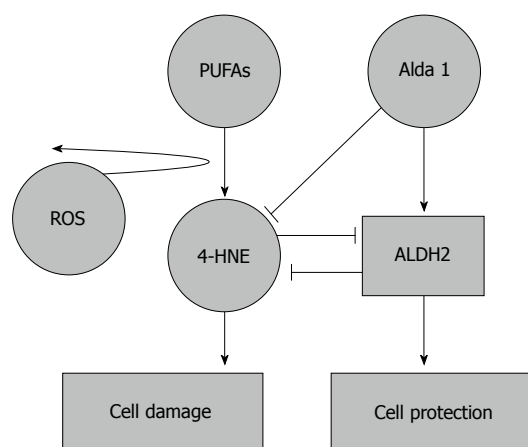
Figure 1 Protective effects of aldehyde dehydrogenase 2 on 4-hydroxy-2-nonenal accumulation in ischemia reperfusion injury. 4-hydroxy-2-nonenal (4-HNE) is a pivotal marker for cell damage associated with oxidative stress; its accumulation is prevented by aldehyde dehydrogenase 2 (ALDH2) activation and the action of its agonists. An overwhelming 4-HNE accumulation may also inhibit ALDH2 action. 4-HNE: 4-hydroxy-2-nonenal.

exposing the cell to a high level of stress and finally leading to its death. In this regard, it is clear that 4-HNE may modify essential cell-signaling molecules of survival mechanisms, as it does in autophagy (AMPK) and apoptosis (Akt)[27,28,30].

Ultimately, the clearance ratio of 4-HNE-adducts by ALDH2 and the ALDH2-4-HNE balance is crucial in order to prevent the modulation of survival mechanisms by 4-HNE and thus minimize some of the deleterious effects of IRI caused by the formation of these toxic aldehydes (Figure 1).

ALDH2 AND AUTOPHAGY

Autophagy is believed to play a key role in cell survival, with distinct functions depending on the event the cell is facing. In fact, autophagy is a cellular housekeeping mechanism, which in physiological conditions removes long-lived, aggregated and misfolded proteins, clears damaged organelles and plays an important role in the "survival and death" strategies of cellular stress responses, as occurs in the I/R condition[31,32-34].

Autophagy has two paradoxically opposite behaviors, depending on the stage of the I/R condition: during ischemia, certain levels of autophagy are cytoprotective[35], but during reperfusion it is detrimental[32,33]. One of the key elements that regulate autophagy is the mammalian target of rapamycin (mTOR), an autophagy inhibitor. The inhibitory effect of mTOR over autophagy can be enhanced by Akt during reperfusion, or inhibited by AMPK (in both ischemia and reperfusion)[31]. Taking into account the dual behavior of autophagy and seeing some of its regulators, the role that ALDH2 plays in I/R is also dual: During ischemia, ALDH2 benefits AMPK activation, thus contributing to the inhibition of mTOR and subsequently increasing cytoprotective autophagy. On the other hand, during the reperfusion

phase, cytoprotective autophagy is no longer active. Under reperfusion conditions, ALDH2 inhibits AMPK but also activates Akt, whose phosphorylation is kicked on to activate mTOR, thus resulting in a detrimental Akt-dependent inhibition of autophagy[28]. The ALDH2 dual regulatory effect on autophagy mechanisms through the Akt/AMPK/mTOR pathway could be a useful tool for preventing IRI. Considering that both AMPK and Akt are targets for 4-HNE, the beneficial effects that ALDH2 exerts on autophagy may be due both to its interaction with AMPK and to its capacity to remove 4-HNE (Figure 2).

We have seen that autophagy is a mechanism which, during periods of scarce resources, enables the cell to recycle its own products in order to survive. Most of this survival potential is performed through the preservation of the mitochondria, which are among the organelles most in need of protection. Therefore, mitophagy, the selective process to auto-phagocytize mitochondria, should be carefully regulated[36]. Some IRI-related studies report that after ALDH2 activation, the mitophagy regulators tensin homolog-induced putative kinase 1 (PINK1)/Parkin expression is suppressed, resulting in reduced injury. Even though the specific mechanism of action remains unclear (either *via* direct inhibition or *via* 4-HNE cleansing), ALDH2 clearly exerts a beneficial effect on mitophagy[37].

We can conclude that ALDH2 plays a positive role in the modulation of cyto-protective autophagy. It is an especially promising agent for the regulation of the deleterious effects of autophagy in cold IRI processes associated with organ transplantation.

ALDH2: NECROPTOSIS AND APOPTOSIS

Programmed cell death results in either a lytic or a non-lytic morphology, which includes different cell signaling pathways. It can be caused by well-known mechanisms such as autophagy, apoptosis, necrosis and necroptosis[19,38,39], which are determinant for organ preservation/cell survival associated with ischemia reperfusion injury.

Briefly, "necrosis" is one of the most frequent consequences of metabolic injury due to the organ oxygen deprivation during ischemia, leading to ATP depletion. It is characterized by cell swelling, membrane rupture and release of cell contents with the subsequent inflammatory response[40]. By contrast, "apoptosis" is a non-lytic form of silent programmed cell death in which the individual dying cells separate from their neighbors and shrink rather than swell[40]. Cell death by necrosis may also be programmed, as also occurs in apoptosis, and is then called "necroptosis". In any case, distinct signaling events drive the lytic cell and non-lytic cell death processes (necroptosis and apoptosis, respectively). Thus, necroptosis is similar in nature to necrosis but it is induced by the activation of RIPK1 (receptor-interacting protein kinase 1) and RIPK3
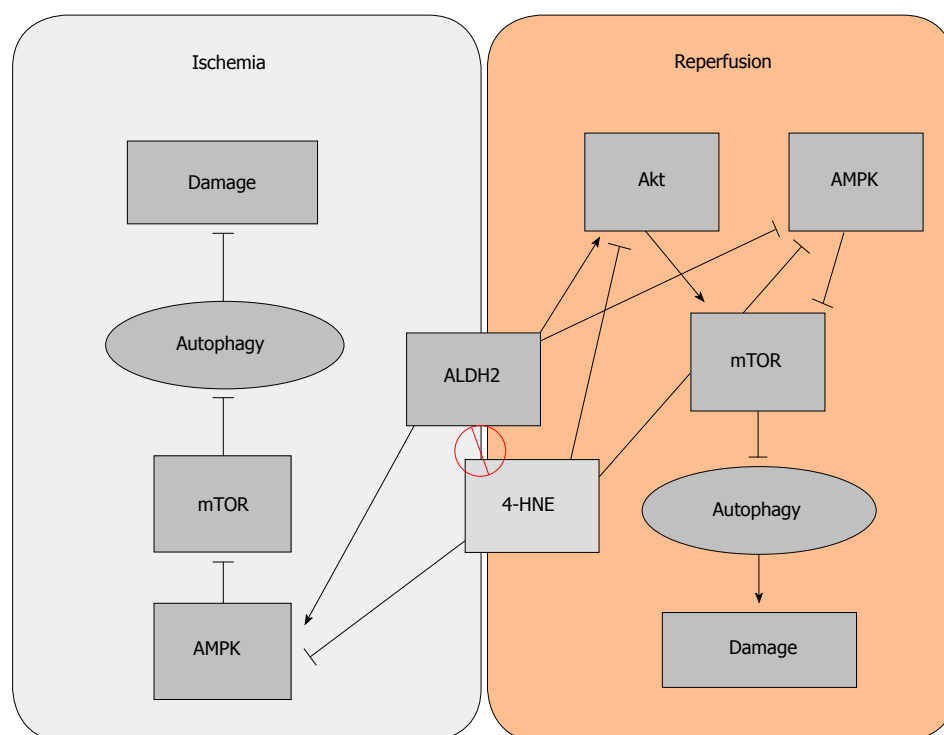
Figure 2 Dual regulatory effect of aldehyde dehydrogenase 2 (ALDH2) on autophagy. During ischemia, AMPK is activated to promote "cytoprotective" autophagy due to mTOR inhibition. On the other hand, the activation of Akt during the phase of reperfusion inhibits "deleterious" autophagy, which is associated with apoptosis, thus reducing organ damage.

(receptor- interacting protein kinase 1), a caspase-independent form of programmed cell death. Studies by Cheng Shen *et al*[41] showed that ALDH2 deficiency promotes necroptosis through the activation of the RIPK1/RIPK3/MLKL pathway.

In contrast to necroptosis, there is no inflammation during apoptosis; the process is dependent on caspases 3 and 9. Apoptosis is a process controlled at multiple checkpoints by cell signaling expression, sometimes with opposite actions; examples are Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic). The ratio between the two is decisive for the apoptotic response, which is regulated by an upstream mediator, Akt, which ultimately determines the fate of the cell[42,43].

Due to its cytotoxic characteristics, 4-HNE is an important mediator of oxidative stress-induced apoptosis[42]. As previously mentioned, 4-HNE inhibits a group of a several kinases, one of which is Akt. Under high concentrations of 4-HNE, a dramatic apoptotic response is induced. This effect has been suggested to occur through the inhibition of Akt, which increases the Bax/Bcl-2 ratio and activates a caspase-3 apoptotic cascade[43,44]. This Akt inhibition in the presence of 4-HNE can be totally reversed by using an ALDH2 agonist, such as Alda-1[45]. Further investigations have evidenced that increased ALDH2 expression through Alda-1 treatment protects I/R-induced brain cell necrosis and apoptosis[16,24]. Besides these benefits of ALDH2 observed in the heart and brain, benefits have also been shown for limiting neuronal apoptosis in spinal cord

IRI[24]. Recently, Zhong *et al*[45] reported that low ALDH2 promotes liver apoptosis through the MAPK pathway when ALDH2 agonists are used, in which ALDH2 action is not only based on the 4-HNE clearance ratio, but also on its subsidiary involvement in controlling indirect 4-HNE pathways.

Again, the ratio of 4-HNE formation and its cleansing by ALDH2 is essential. High 4-HNE levels will limit ALDH2 activity, avoiding subsequent 4-HNE clearance and producing a positive 4-HNE feedback that ultimately causes apoptosis and cell death[1,42] (Figure 3).

ALDH2 ACTIVATION AND IRI PREVENTION IN DIFFERENT ORGANS: SOME CONSIDERATIONS

In general, ALDH2 activation plays a protective role in the complex physiopathology of IRI, whose beneficial effects have mostly been studied in the heart, inducing cardio-protection as well as a protective role against the I/R insult[12,46]. This protection has been evidenced in other organs such as brain, intestine, kidney and spinal cord[16,20,22,24]. Surprisingly, the role of ALDH2 in hepatic I/R has not been studied in depth, even though the liver is one of the organs where ALDH2 is most abundant[1]. This is probably due to the fact that most studies of ALDH2 in liver have focused on ethanol metabolism disorders.
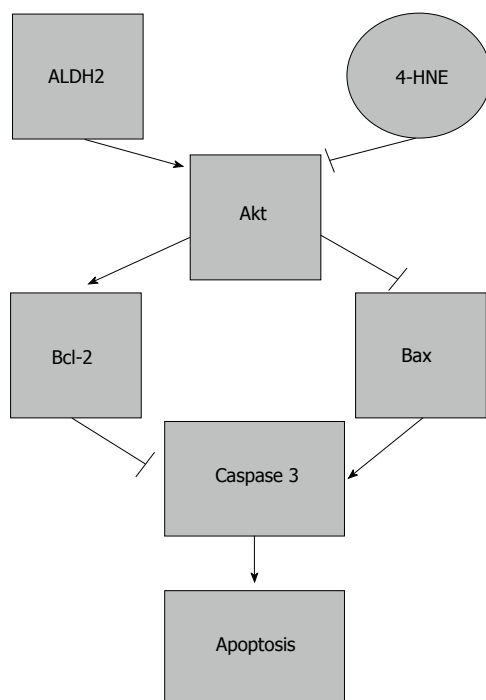
In the following lines, we describe the most

Figure 3 Effects of aldehyde dehydrogenase 2 on apoptosis in ischemia reperfusion injury. Protection induced by aldehyde dehydrogenase 2 (ALDH2) on apoptosis. The balance between ALDH2/4-HNE is responsible for the modulation of apoptosis through Akt and caspase 3 expression.

important ongoing research into the role of ALDH2 in IRI in heart, brain, eyes, intestine, and kidney.

ALDH2 and heart
The most important feature of ALDH2 myocardial cardio-protection is the clearance of toxic aldehydes (such as 4-HNE) and its adducts[12,46]; Alda-1, an activator of ALDH2, raises this reactive aldehyde clearance even further, increasing the protection against myocardial IRI disorders[14,15]. Moreover, ALDH2 activation is associated with an improved mitochondrial function and a remodeling of the ventricular function[47,48]. ALDH2-induced cardio-protection (with the subsequent detoxification of toxic aldehydes) against IRI also occurs through a differential regulation of autophagy, involving both AMPK and Akt-mTOR signaling mechanisms, produced during ischemia and reperfusion respectively[28,49]. Guo *et al*[50] recently described a novel protective ALDH2 mechanism in type I diabetes, which defends against the induced myocardial dysfunction *via* an AMPK-dependent regulation of autophagy. These benefits are confirmed by the fact that ALDH2 inhibition by O-linked-N-acetylglucosamine (O-GlcNAC) acylation contributes to hyperglycemic exacerbation of myocardial IRI, which is prevented by Alda-1[51]. Even more recently, it has been reported that ALDH2 induces cardio-protection through the regulation of mitophagy by suppressing tensin homolog-induced putative kinase 1 (PINK1)/Parkin expression, thus preventing the accumulation of 4-HNE and other toxic species[37].

ALDH2 in brain, intestine, kidney and eyes
The role of ALDH2 in other organs, such as brain, eyes, intestine, and kidney, has been poorly investigated. In brain and intestine, ALDH2 activation contributes to the clearance of the 4-HNE accumulation and MDA generation against IRI. Alda-1, an ALDH2 agonist, increases the 4-HNE adduct clearance, subsequently promoting protection against brain IRI and preventing the deleterious effects of apoptosis[15,16]. In kidney, after bilateral ischemia, ALDH2 activation is involved in the protection induced by ethanol at physiological levels[23]. Interestingly, an increased ALDH2 expression also has a protective effect, reducing renal cell apoptosis by inhibiting the MAPK pathway, after using hypothermic machine perfusion[22].

Recent investigations have shown the existence of ALDH2 expression in adult rat retinal tissues, which may be involved in the retina redox balance. However, its role in the regulatory lipoperoxidation mechanisms associated with retinal ischemia reperfusion injury needs to be investigated in depth[52,53].

ALDH2 ACTIVATION: ISCHEMIC PRECONDITIONING
Several therapeutic surgical and pharmacological strategies have been used to protect the organ against I/R insult. As an example, "ischemic preconditioning" (IPC) is based on the application of a previous transient ischemia and/or reperfusion that will prepare the organ prior to a sustained I/R. IPC was initially evidenced in the heart by Murry *et al*[54] but has also been found to be protective for other organs such as kidney, intestine and liver[55-57]. Another surgical strategy is "remote ischemic preconditioning" (RIPC)[58], which consists in the application of brief ischemia in one organ to confer protection on distant organs as well[59]. The involvement of ALDH2 as an important mediator in the benefits conferred by RIPC has also been discussed by Contractor *et al*[59].

ALDH2 ACTIVATION: PHARMACOLOGICAL PRECONDITIONING
The use of pharmacological agents to promote organ protection (pharmacological preconditioning) is limited when it is mandatory to avoid their potential undesirable secondary effects. We have assessed the protective role of ALDH2 when using different protective strategies against IRI based on the administration of volatile anesthetics (isoflurane), nitrites/nitrates and ALDH2 agonists (Alda-1).

ALDH2 and isoflurane preconditioning
Recent investigations in the heart and other organs have demonstrated that the use of isoflurane, a volatile anesthetic agent, is an effective preconditioning agent

that mimics the protective effects of IPC but avoids some of its disadvantages caused by the reduction of the blood flow, and presents greater ethical acceptability and clinical safety[60-62]. ALDH2 is involved in the cardio-protection induced by isoflurane preconditioning, and this ALDH2/isoflurane-induced cardio-protection is substantially blocked by a PKCε inhibitor, suggesting that mitochondrial PKCε plays an important role in isoflurane-induced protection mechanisms. It has been discussed whether the phosphorylation of ALDH2 increases PKCε mitochondrial translocation, and the inhibition of mitochondrial translocation by other protein kinases such as PKC delta, contribute even more to isoflurane protection against I/R insult[63].

ALDH2 and ethanol preconditioning

Ethanol, at low doses, also acts as a preconditioning agent[51]. Its acute cardio-protective effects are critically modulated by the dose used and by whether it remains present during the ischemic period. In these conditions of ethanol preconditioning, ALDH2 is activated to induce protection against IRI through the mitochondrial translocation of PKCε, which is responsible for the increased metabolism from HNE to HNA, thus limiting the accumulation of HNE protein adducts and improving cardiac function[64,65].

In this context, it has been suggested that the use of ALDH2 activators mimics ethanol cardio-protection. In addition, ALDH2 protection against ethanol toxicity is regulated by Akt and AMPK, and subsequently, autophagy and apoptosis through their downstream substrate mTOR[28].

ALDH2 and NO/nitrite preconditioning

Nitric oxide (NO) is a free-radical gas, considered as a relevant therapeutic tar- get during IRI. Its effects on I/R injury are probably related to the dose or to the conditions during ischemia and reperfusion, usually at nanomolar or low micromolar concentrations[66]. Apart from its role in tissue protection during ischemia, and due to its volatile nature, NO is a fast-response cell signaling molecule and a well-known vasodilator during hypoxic events, also regulating mitochondrial oxygen consumption[66]. There is growing evidence that eNOS-derived NO is a critical component in IPC and APC signal transduction[67,68]. Last but not least, due to its reductive capacity, NO is a potent ROS scavenger, and thus, performs one of the most important functions during reperfusion[69].

As a volatile molecule, the half-life of NO is not very long; this is why there is a need for a pool of nitrogen in a more stable form, i.e., nitrites (NO_2-) and nitrates (NO_3-). Nitrites are the reduced form of nitrates, and under physiological conditions they can be recycled in blood and tissues, where NO generation from nitrites is linearly dependent on oxygen and pH levels[66,69].

It has been widely shown that ALDH2 has a reductase-dependent activity that produces the

bioconversion of nitroglycerin to 1, 2-glyceryl denigrates, resulting in the release of NO[1]. Further experiments have shown that in vitro, ALDH2 is partially responsible for nitrite bioactivation, promoting vasodilation during hypoxic episodes (Figure 4). However, Oelze et al[70] showed that the peroxynitrite generated from NO impairs the enzymatic activity of ALDH2, and therefore the scavenging activity of NO may result in a suicide inhibition of ALDH2 in later stages.

The role of ALDH2 cannot be conclusively confirmed, due to the impossibility of ruling out other non- enzymatic or nitrite reductase species (e.g., eNOS)[71-73]. However, it can be concluded that ALDH2 is closely related to NO and nitrogen metabolism and exerts positive effects[72,73].

ALDH2 agonist preconditioning: Alda-1

With this in mind, it has been proposed that new therapeutic ALDH2 approaches might be based on promoting ALDH2 over-expression by using ALDH2 activators and determining the role of ALDH2 in a variety of human pathologies such as IRI. Here we focus specifically on the use of Alda-1, a selective ALDH2 agonist, in IRI in different organs.

Small molecules called ALDAs have been used as activators of ALDH2. One of the most frequently used is Alda-1. Defined as N-(1,3-benzodioxol-5-ylmethyl)-2,6 di-chloro-benzamide, Alda-1 increases ALDH2 activity in humans twofold with its wild-type and 11-fold with its defective variant ALDH2*2[1].

Experiments have shown that the administration of Alda-1 just before the ischemic insult reduces infarct size by 60%, most likely through ALDH2 activation[12]. Alda-1 also has beneficial effects on the heart and brain by inhibiting 4-HNE and related adducts[15]. A potential therapeutic value of Alda-1 during the progression of post-myocardial infarction cardiomyopathy has also been suggested[29]. Alda-1 may be useful for treating heart failure patients, since ALDH2 activation in heart failure restores mitochondrial function and improves ventricular function and remodeling[48]. It was recently reported that ALDH2 activation regulates mitophagy by preventing 4-HNE, ROS and superoxide dismutase (SOD) accumulation[37]. Along these lines, the recent investigations by Zhu et al[20] have shown that Alda-1 pre-treatment reduces the intestinal injury induced by I/R in mice. Benefits were associated with the prevention of 4-HNE and MDA accumulation, suggesting a potential clinical application in the near future[21].

FUTURE PERSPECTIVES OF ALDH2 IN COLD ISCHEMIA REPERFUSION INJURY/ ORGAN TRANSPLANTATION

ROS play a major role in the progression of IRI, which is inherent to organ transplantation. ROS are responsible for the generation of MDA, as well as other toxic aldehydes
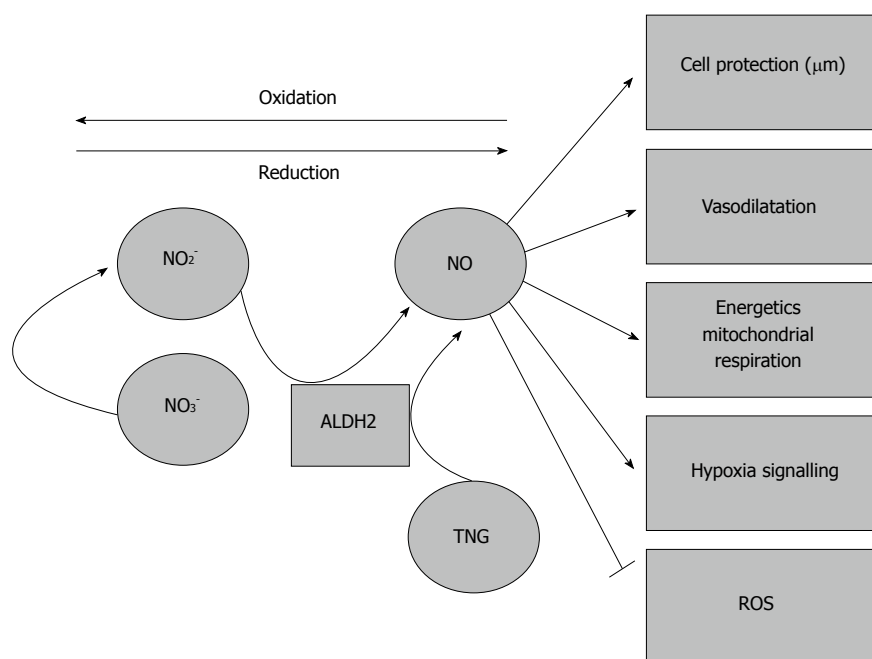
Figure 4 Aldehyde dehydrogenase 2 and vasodilation due to nitrates/nitrites/nitric oxide generation. The generation of nitric oxide from nitrates/nitrites and its vasodilatory properties may contribute to prevent the deleterious effects of ischemia-reperfusion injury. ALDH2: Aldehyde dehydrogenase 2.

like 4-HNE and its adducts. During graft reperfusion, ROS are mainly produced in the mitochondria, becoming the main source of lipoperoxides and toxic aldehydes. Therefore, the prevention and clearance of those toxic elements generated during an episode of ischemia-reperfusion is decisive in organ transplantation, and the role of ALDH2 is crucial.

So far, the vast majority of investigations on the role of ALDH2 activation in organ preservation are limited to the heart and kidney[22,74]. Gong *et al*[74] demonstrated that addition of Alda-1 (ALDH2 activator) to histidine-ketoglutarate-tryptophan (HTK; a solution regularly used in heart surgery) improved cardio-protection through the subsequent ALDH2 activation and toxic aldehyde removal. The fact that Alda-1 ameliorates the quality of cardioplegic solutions such as HTK suggests that ALDH2 activators could be used to improve preservation solutions meant for other organs besides the heart, such as UW, HTK, Celsior and IGL-1, which are used for clinical transplantation in liver, where ALDH2 has an important presence[75].

These findings have a bearing on the consideration of the potential use of ALDH2 activators as additives in organ preservation solutions in order to improve their protective quality. They are also relevant to the assessment of the contribution of various commercial solutions to the preservation of mitochondrial ALDH2 activity during graft cold storage, especially when expanded criteria donors are used.

Recent studies have reported higher mitochondrial ALDH2 activity in liver grafts preserved in IGL-1 (24 h at 4 ℃) than in livers preserved in UW or HTK solutions[76]. These results are consistent with the increases reported

in renal ALDH2 activity by Zhong *et al*[22], who subjected kidney grafts to machine perfusion preservation before transplantation.

These results confirm that ALDH2 plays a role in both static and dynamic graft preservation. It exerts a protective effect, during the ischemic phase by tackling ATP breakdown and during the reperfusion phase by reducing oxidative damage, which are inherent conditions of IRI. In this regard, further studies of ALDH2 using machine perfusion strategies in subnormothermic conditions should be carried out in order to improve the subsequent graft outcome.

Finally, although ALDH2 plays a role in distinct organs with well-defined physiological functions (heart, brain, eyes, liver, kidney and intestine), each organ contains multiple different cell types which, in turn, may give specific responses under ALDH2 activation/administration. Few studies have sought to clarify the relationship between ALDH2 and specific cell types or its influence on the substantial cross-talk between the different cells in each organ under ALDH2 administration[77]. Therefore, further investigation of this issue is now warranted.

CONCLUSION

In recent years, the ability of ALDH2 to modify the activity of some key enzymes and essential survival pathways of the cell has been demonstrated (autophagy, apoptosis, necroptosis, *etc*). Some of its effects are exerted directly through its catalytic center, in a one-to-one interaction with those key enzymes that modifies the whole metabolic pathway. However, it may also regulate the metabolism indirectly, removing and cleansing toxic
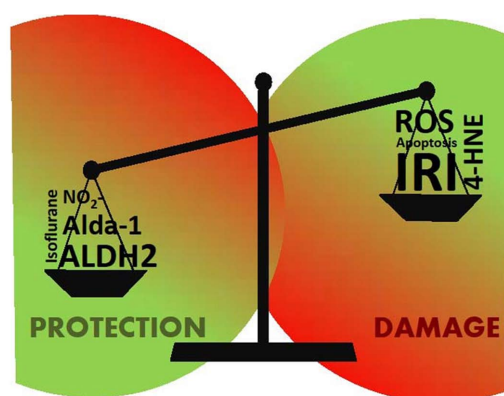
Figure 5 Aldehyde dehydrogenase 2 and 4-hydroxy-2-nonenal balance in ischemia reperfusion injury. Red areas represent damage, whereas green ones depict protection of the cell through the various mechanisms. ALDH2: Aldehyde dehydrogenase 2; 4-HNE: 4-hydroxy-2-nonenal.

sub-products resulting from pathologies like IRI, which compromise cell viability, as we have seen in the case of 4-HNE (Figure 5).

As a result, ALDH2 will affect any metabolic pathway in which 4-HNE is involved, and so its importance lies not only in its ability to activate other enzymes, but also in the collateral effects of those metabolic processes regulated by 4-HNE. In these processes, the regulation levels will depend on the balance of several factors such as the aldehyde/ALDH2 production ratio (where ALDH2 agonists take on a major role), the metabolization ratio of 4-HNE by ALDH2 and whether 4-HNE reaches the level of no-return where it inhibits all the ALDH2 present and the balance is definitely broken (Figure 5).

ALDH2 affects a wide range spectrum of mechanisms, among which ethanol metabolism has been the most studied. Yet, its role in important pathophysiological processes, such as IRI, remains unclear. With regard to IRI, the behavior of ALDH2 differs according to the phase (ischemia or reperfusion). Given that a variety of harmful elements such as 4-HNE are produced during an I/R episode, ALDH2 may play a critical role in the liver. This role should be further elucidated in future studies.

However, just as most studies have focused on the role of ALDH2 in ethanol metabolism rather than IRI, studies of ALDH2 in IRI are mainly focused on heart, brain or kidney and have paid hardly any attention to the liver. This is especially surprising in view of the fact that the liver is the organ with the highest ALDH2 concentration recorded so far due its detoxifying role. Just before this review was completed, a study evidencing the protective role of ALDH2 activation by Alda-1 in a model of warm ischemia reperfusion in liver was published[78].

ACKNOWLEDGMENTS

REFERENCES

1   **Chen CH**, Ferreira JC, Gross ER, Mochly-Rosen D. Targeting aldehyde dehydrogenase 2: new therapeutic opportunities. *Physiol Rev* 2014; **94**: 1-34 [PMID: 24382882 DOI: 10.1152/physrev.00017.2013]

2   **Klyosov AA**, Rashkovetsky LG, Tahir MK, Keung WM. Possible role of liver cytosolic and mitochondrial aldehyde dehydrogenases in acetaldehyde metabolism. *Biochemistry* 1996; **35**: 4445-4456 [PMID: 8605194 DOI: 10.1021/bi9521093]

3   **Klyosov AA**. Kinetics and specificity of human liver aldehyde dehydrogenases toward aliphatic, aromatic, and fused polycyclic aldehydes. *Biochemistry* 1996; **35**: 4457-4467 [PMID: 8605195 DOI: 10.1021/bi9521102]

4   **Mali VR**, Palaniyandi SS. Regulation and therapeutic strategies of 4-hydroxy-2-nonenal metabolism in heart disease. *Free Radic Res* 2014; **48**: 251-263 [PMID: 24237196 DOI: 10.3109/10715762.2013.864761]

5   **Schneider C**, Porter NA, Brash AR. Routes to 4-hydroxynonenal: fundamental issues in the mechanisms of lipid peroxidation. *J Biol Chem* 2008; **283**: 15539-15543 [PMID: 18285327 DOI: 10.1074/jbc.R800001200]

6   **Milkovic L**, Cipak Gasparovic A, Zarkovic N. Overview on major lipid peroxidation bioactive factor 4-hydroxynonenal as pluripotent growth-regulating factor. *Free Radic Res* 2015; **49**: 850-860 [PMID: 25532703 DOI: 10.3109/10715762.2014.999056]

7   **Kristal BS**, Park BK, Yu BP. 4-Hydroxyhexenal is a potent inducer of the mitochondrial permeability transition. *J Biol Chem* 1996; **271**: 6033-6038 [PMID: 8626387]

8   **Kruman I**, Bruce-Keller AJ, Bredesen D, Waeg G, Mattson MP. Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. *J Neurosci* 1997; **17**: 5089-5100 [PMID: 9185546]

9   **Zhang Y**, Ren J. ALDH2 in alcoholic heart diseases: molecular mechanism and clinical implications. *Pharmacol Ther* 2011; **132**: 86-95 [PMID: 21664374 DOI: 10.1016/j.pharmthera.2011.05.008]

10   **Zhang Y**, Babcock SA, Hu N, Maris JR, Wang H, Ren J. Mitochondrial aldehyde dehydrogenase (ALDH2) protects against streptozotocin-induced diabetic cardiomyopathy: role of GSK3β and mitochondrial function. *BMC Med* 2012; **10**: 40 [PMID: 22524197 DOI: 10.1186/1741-7015-10-40]

11   **Hao PP**, Chen YG, Wang JL, Wang XL, Zhang Y. Meta-analysis of aldehyde dehydrogenase 2 gene polymorphism and Alzheimer's disease in East Asians. *Can J Neurol Sci* 2011; **38**: 500-506 [PMID: 21515512]

12   **Chen CH**, Budas GR, Churchill EN, Disatnik MH, Hurley TD, Mochly-Rosen D. Activation of aldehyde dehydrogenase-2 reduces ischemic damage to the heart. *Science* 2008; **321**: 1493-1495 [PMID: 18787169 DOI: 10.1126/science.1158554]

13   **Pang JJ**, Barton LA, Chen YG, Ren J. Mitochondrial aldehyde dehydrogenase in myocardial ischemia-reperfusion injury: from bench to bedside. *Sheng Li Xue Bao* 2015; **67**: 535-544 [PMID: 26701629]

14   **Budas GR**, Disatnik MH, Mochly-Rosen D. Aldehyde dehydrogenase 2 in cardiac protection: a new therapeutic target? *Trends Cardiovasc Med* 2009; **19**: 158-164 [PMID: 20005475 DOI: 10.1016/j.tcm.2009.09.003]

15   **Luo XJ**, Liu B, Ma QL, Peng J. Mitochondrial aldehyde dehydrogenase, a potential drug target for protection of heart and brain from ischemia/reperfusion injury. *Curr Drug Targets* 2014; **15**: 948-955 [PMID: 25163552 DOI: 10.2174/1389450115666140828142401]

16   **Fu SH**, Zhang HF, Yang ZB, Li TB, Liu B, Lou Z, Ma QL, Luo XJ,

Peng J. Alda-1 reduces cerebral ischemia/reperfusion injury in rat through clearance of reactive aldehydes. *Naunyn Schmiedebergs Arch Pharmacol* 2014; **387**: 87-94 [PMID: 24081521 DOI: 10.1007/s00210-013-0922-8]

17   **Qi Y**, Zhao M, Bai Y, Huang L, Yu W, Bian Z, Zhao M, Li X. Retinal ischemia/reperfusion injury is mediated by Toll-like receptor 4 activation of NLRP3 inflammasomes. *Invest Ophthalmol Vis Sci.* 2014; **55**: 5466-5475 [DOI: 10.1167/iovs.14-14380]

18   **Baker ML**, Hand PJ, Wang JJ, Wong TY. Retinal signs and stroke: revisiting the link between the eye and brain. *Stroke* 2008; **39**: 1371-1379 [DOI: 10.1161/STROKEAHA.107.496091]

19   **Gao S,** Andreeva K, Cooper NG. Ischemia-reperfusion injury of the retina is linked to necroptosis via the ERK1/2-RIP3 pathway. *Mol Vis* 2014; **20**: 1374-1387

20   **Zhu Q**, He G, Wang J, Wang Y, Chen W. Pretreatment with the ALDH2 agonist Alda-1 reduces intestinal injury induced by ischaemia and reperfusion in mice. *Clin Sci* (Lond) 2017; **131**: 1123-1136 [PMID: 28325855 DOI: 10.1042/CS20170074]

21   **Dollé L**. Pharmacological enrollment of aldehyde dehydrogenase modulators to assist treating ischemia reperfusion-induced intestinal injury: is there a gap to be bridged? *Clin Sci* (Lond) 2017; **131**: 1137-1140 [PMID: 28533269 DOI: 10.1042/CS20170163]

22   **Zhong Z**, Hu Q, Fu Z, Wang R, Xiong Y, Zhang Y, Liu Z, Wang Y, Ye Q. Increased Expression of Aldehyde Dehydrogenase 2 Reduces Renal Cell Apoptosis During Ischemia/Reperfusion Injury After Hypothermic Machine Perfusion. *Artif Organs* 2016; **40**: 596-603 [PMID: 26582147 DOI: 10.1111/aor.12607]

23   **Yuan Q**, Hong S, Han S, Zeng L, Liu F, Ding G, Kang Y, Mao J, Cai M, Zhu Y, Wang QX. Preconditioning with physiological levels of ethanol protect kidney against ischemia/reperfusion injury by modulating oxidative stress. *PLoS One* 2011; **6**: e25811 [PMID: 22022451 DOI: 10.1371/journal.pone.0025811]

24   **Liu XZ**, Sun X, Shen KP, Jin WJ, Fu ZY, Tao HR, Xu ZX. Aldehyde dehydrogenase 2 overexpression inhibits neuronal apoptosis after spinal cord ischemia/reperfusion injury. *Neural Regen Res* 2017; **12**: 1166-1171 [PMID: 28852401 DOI: 10.4103/1673-5374.211198]

25   **Casillas-Ramírez A**, Mosbah IB, Ramalho F, Roselló-Catafau J, Peralta C. Past and future approaches to ischemia-reperfusion lesion associated with liver transplantation. *Life Sci* 2006; **79**: 1881-1894 [PMID: 16828807 DOI: 10.1016/j.lfs.2006.06.024]

26   **Zaouali MA**, Ben Abdennebi H, Padrissa-Altés S, Mahfoudh-Boussaid A, Roselló-Catafau J. Pharmacological strategies against cold ischemia reperfusion injury. *Expert Opin Pharmacother* 2010; **11**: 537-555 [PMID: 20163266 DOI: 10.1517/14656560903547836]

27   **Mali VR**, Deshpande M, Pan G, Thandavarayan RA, Palaniyandi SS. Impaired ALDH2 activity decreases the mitochondrial respiration in H9C2 cardiomyocytes. *Cell Signal* 2016; **28**: 1-6 [PMID: 26577527 DOI: 10.1016/j.cellsig.2015.11.006]

28   **Ma H**, Guo R, Yu L, Zhang Y, Ren J. Aldehyde dehydrogenase 2 (ALDH2) rescues myocardial ischaemia/reperfusion injury: role of autophagy paradox and toxic aldehyde. *Eur Heart J* 2011; **32**: 1025-1038 [PMID: 20705694 DOI: 10.1093/eurheartj/ehq253]

29   **Gomes KM**, Bechara LR, Lima VM, Ribeiro MA, Campos JC, Dourado PM, Kowaltowski AJ, Mochly-Rosen D, Ferreira JC. Aldehydic load and aldehyde dehydrogenase 2 profile during the progression of post-myocardial infarction cardiomyopathy: benefits of Alda-1. *Int J Cardiol* 2015; **179**: 129-138 [PMID: 25464432 DOI: 10.1016/j.ijcard.2014.10.140]

30   **Liu W**, Akhand AA, Takeda K, Kawamoto Y, Itoigawa M, Kato M, Suzuki H, Ishikawa N, Nakashima I. Protein phosphatase 2A-linked and -unlinked caspase-dependent pathways for downregulation of Akt kinase triggered by 4-hydroxynonenal. *Cell Death Differ* 2003; **10**: 772-781 [PMID: 12815460 DOI: 10.1038/sj.cdd.4401238]

31   **Van Erp AC**, Hoeksma D, Rebolledo RA, Ottens PJ, Jochmans I, Monbaliu D, Pirenne J, Leuvenink HGD, Decuypere JP. The Crosstalk between ROS and Autophagy in the Field of Transplantation Medicine. *Oxid Med Cell Longev* 2017; **2017**: 7120962 [PMID: 29410735 DOI: 10.1155/2017/7120962]

32   **Decuypere JP**, Ceulemans LJ, Agostinis P, Monbaliu D, Naesens M, Pirenne J, Jochmans I. Autophagy and the Kidney: Implications for Ischemia-Reperfusion Injury and Therapy. *Am J Kidney Dis* 2015; **66**: 699-709 [PMID: 26169721 DOI: 10.1053/j.ajkd.2015.05.021]

33   **Cursio R**, Colosetti P, Gugenheim J. Autophagy and liver ischemia-reperfusion injury. *Biomed Res Int* 2015; **2015**: 417590 [PMID: 25861623 DOI: 10.1155/2015/417590]

34   **Go KL**, Lee S, Zendejas I, Behrns KE, Kim JS. Mitochondrial Dysfunction and Autophagy in Hepatic Ischemia/Reperfusion Injury. *Biomed Res Int* 2015; **2015**: 183469 [PMID: 26770970 DOI: 10.1155/2015/183469]

35   **Panisello-Roselló A**, Verde E, Lopez A, Flores M, Folch-Puy E, Rolo A, Palmeira C, Hotter G, Carbonell T, Adam R, Roselló-Catafau J. Cytoprotective Mechanisms in Fatty Liver Preservation against Cold Ischemia Injury: A Comparison between IGL-1 and HTK. *Int J Mol Sci* 2018; **19** [PMID: 29364854 DOI: 10.3390/ijms19020348]

36   **Poole AC**, Thomas RE, Andrews LA, McBride HM, Whitworth AJ, Pallanck LJ. The PINK1/Parkin pathway regulates mitochondrial morphology. *Proc Natl Acad Sci* USA 2008; **105**: 1638-1643 [PMID: 18230723 DOI: 10.1073/pnas.070933610]

37   **Ji W**, Wei S, Hao P, Xing J, Yuan Q, Wang J, Xu F, Chen Y. Aldehyde Dehydrogenase 2 Has Cardioprotective Effects on Myocardial Ischaemia/Reperfusion Injury via Suppressing Mitophagy. *Front Pharmacol* 2016; **7**: 101 [PMID: 27148058 DOI: 10.3389/fphar.2016.00101]

38   **Wang S**, Pacher P, De Lisle RC, Huang H, Ding WX. A Mechanistic Review of Cell Death in Alcohol-Induced Liver Injury. *Alcohol Clin Exp Res* 2016; **40**: 1215-1223 [DOI: 10.1111/acer.13078]

39   **Rosenbaum DM,** Degterev A, David J, Rosenbaum PS, Roth S, Grotta JC, Cuny GD, Yuan J, Savitz SI. Necroptosis, a novel form of caspase-independent cell death, contributes to neuronal damage in a retinal ischemia-reperfusion injury model. *J Neurosci Res* 2010; **88**: 1569-1576 [DOI: 10.1002/jnr.22314]

40   **Malhi H,** Gores GJ, Lemasters JJ. Apoptosis and necrosis in the liver: a tale of two deaths? *Hepatology* 2006**; 43**: S31-S44

41   **Shen C,** Wang C, Han S, Wang Z, Dong Z, Zhao X, Wang P, Zhu H, Sun X, Ma X, Zhu H, Zou Y, Hu K, Ge J, Sun A. Aldehyde dehydrogenase 2 deficiency negates chronic low-to-moderate alcohol consumption-induced cardioprotecion possibly via ROS-dependent apoptosis and RIP1/RIP3/MLKL-mediated necroptosis. *Biochim Biophys* Acta 2017; **1863**: 1912-1918 [DOI: 10.1016/j.bbadis.2016.11.016]

42   **Awasthi YC**, Sharma R, Cheng JZ, Yang Y, Sharma A, Singhal SS, Awasthi S. Role of 4-hydroxynonenal in stress-mediated apoptosis signaling. *Mol Aspects Med* 2003; **24**: 219-230 [PMID: 12893000 DOI: 10.1016/S0098-2997(03)00009-8]

43   **Teijido O**, Dejean L. Upregulation of Bcl2 inhibits apoptosis-driven BAX insertion but favors BAX relocalization in mitochondria. *FEBS Lett* 2010; **584**: 3305-3310 [PMID: 20621101 DOI: 10.1016/j.febslet.2010.07.002]

44   **Ji GR**, Yu NC, Xue X, Li ZG. 4-Hydroxy-2-nonenal induces apoptosis by inhibiting AKT signaling in human osteosarcoma cells. *ScientificWorldJournal* 2014; **2014**: 873525 [PMID: 24711740 DOI: 10.1155/2014/873525]

45   **Zhong Z**, Ye S, Xiong Y, Wu L, Zhang M, Fan X, Li L, Fu Z, Wang H, Chen M, Yan X, Huang W, Ko DS, Wang Y, Ye Q. Decreased expression of mitochondrial aldehyde dehydrogenase-2 induces liver injury via activation of the mitogen-activated protein kinase pathway. *Transpl Int* 2016; **29**: 98-107 [PMID: 26404764 DOI: 10.1111/tri.12675]

46   **Liu X**, Sun A. Aldehyde Dehydrogenase-2 Roles in Ischemic Cardiovascular Disease. *Curr Drug Targets* 2017; **18**: 1817-1823 [PMID: 27633387 DOI: 10.2174/1389450117666160912174417]

47   **Mali VR**, Pan G, Deshpande M, Thandavarayan RA, Xu J, Yang XP, Palaniyandi SS. Cardiac Mitochondrial Respiratory Dysfunction and Tissue Damage in Chronic Hyperglycemia Correlate with Reduced Aldehyde Dehydrogenase-2 Activity. *PLoS One* 2016; **11**: e0163158 [PMID: 27736868 DOI: 10.1371/journal.pone.0163158]

48   **Gomes KM**, Campos JC, Bechara LR, Queliconi B, Lima VM, Disatnik MH, Magno P, Chen CH, Brum PC, Kowaltowski AJ,

Mochly-Rosen D, Ferreira JC. Aldehyde dehydrogenase 2 activation in heart failure restores mitochondrial function and improves ventricular function and remodelling. *Cardiovasc Res* 2014; **103**: 498-508 [PMID: 24817685 DOI: 10.1093/cvr/cvu125]

49   **Zhang Y**, Ren J. Autophagy in ALDH2-elicited cardioprotection against ischemic heart disease: slayer or savior? *Autophagy* 2010; **6**: 1212-1213 [PMID: 20935489]

50   **Guo Y**, Yu W, Sun D, Wang J, Li C, Zhang R, Babcock SA, Li Y, Liu M, Ma M, Shen M, Zeng C, Li N, He W, Zou Q, Zhang Y, Wang H. A novel protective mechanism for mitochondrial aldehyde dehydrogenase (ALDH2) in type i diabetes-induced cardiac dysfunction: role of AMPK-regulated autophagy. *Biochim Biophys Acta* 2015; **1852**: 319-331 [PMID: 24874076 DOI: 10.1016/j.bbadis.2014.05.017]

51   **Liu B**, Wang J, Li M, Yuan Q, Xue M, Xu F, Chen Y. Inhibition of ALDH2 by O-GlcNAcylation contributes to the hyperglycemic exacerbation of myocardial ischemia/reperfusion injury. *Oncotarget* 2017; **8**: 19413-19426 [PMID: 28038474 DOI: 10.18632/oncotarget.14297]

52   **Pinazo-Durán MD,** Verdejo C, Azorín I, Renau-Piqueras J, Iborra FJ. Colocalization of aldehyde dehydrogenases and Fe/NADPH-induced lipid peroxidation in tissue sections of rat retina. *Ophthalmic Res* 2000; **32**: 61-68

53   **Galbis-Estrada C,** Pons-Vázquez S, Gallego-Pinazo R, Lleó-Perez A, Garcia-Medina JJ, Bou VV, Sanz-Solana P, Pinazo-Durán MD. Glutathione-dependent formaldehyde dehydrogenase (ADH3) and low km mitochondrial aldehyde dehydrogenase (ALDH2). New evidence for differential expression in the rat retina in response to oxidative stress. *Free Radic Res* 2012; **46**: 77-84 [DOI: 10.3109/10 715762.2011.640324]

54   **Murry CE**, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986; **74**: 1124-1136 [PMID: 3769170 DOI: 10.1161/01.CIR.74.5.1124]

55   **Riera M**, Herrero I, Torras J, Cruzado JM, Fatjo M, Lloberas N, Alsina J, Grinyo JM. Ischemic preconditioning improves postischemic acute renal failure. *Transplant Proc* 1999; **31**: 2346-2347 [PMID: 10500611 DOI: 10.1016/S0041-1345(99)00372-3]

56   **Sola A**, De Oca J, González R, Prats N, Roselló-Catafau J, Gelpí E, Jaurrieta E, Hotter G. Protective effect of ischemic preconditioning on cold preservation and reperfusion injury associated with rat intestinal transplantation. *Ann Surg* 2001; **234**: 98-106 [PMID: 11420489]

57   **Peralta C**, Bartrons R, Riera L, Manzano A, Xaus C, Gelpí E, Roselló-Catafau J. Hepatic preconditioning preserves energy metabolism during sustained ischemia. *Am J Physiol Gastrointest Liver Physiol* 2000; **279**: G163-G171 [PMID: 10898759 DOI: 10.1152/ajpgi.2000.279.1.G163]

58   **Tapuria N**, Kumar Y, Habib MM, Abu Amara M, Seifalian AM, Davidson BR. Remote ischemic preconditioning: a novel protective method from ischemia reperfusion injury--a review. *J Surg Res* 2008; **150**: 304-330 [PMID: 19040966 DOI: 10.1016/j.jss.2007.12.747]

59   **Contractor H**, Støttrup NB, Cunnington C, Manlhiot C, Diesch J, Ormerod JO, Jensen R, Bøtker HE, Redington A, Schmidt MR, Ashrafian H, Kharbanda RK. Aldehyde dehydrogenase-2 inhibition blocks remote preconditioning in experimental and human models. *Basic Res Cardiol* 2013; **108**: 343 [PMID: 23525499 DOI: 10.1007/s00395-013-0343-3]

60   **Kersten JR**, Schmeling TJ, Pagel PS, Gross GJ, Warltier DC. Isoflurane mimics ischemic preconditioning via activation of K(ATP) channels: reduction of myocardial infarct size with an acute memory phase. *Anesthesiology* 1997; **87**: 361-370 [PMID: 9286901]

61   **Tsutsumi YM**, Patel HH, Lai NC, Takahashi T, Head BP, Roth DM. Isoflurane produces sustained cardiac protection after ischemia-reperfusion injury in mice. *Anesthesiology* 2006; **104**: 495-502 [PMID: 16508397]

62   **Li H**, Lang XE. Protein kinase C signaling pathway involvement in cardioprotection during isoflurane pretreatment. *Mol Med Rep* 2015;

11: 2683-2688 [PMID: 25482108 DOI: 10.3892/mmr.2014.3042]

63   **Lang XE**, Wang X, Zhang KR, Lv JY, Jin JH, Li QS. Isoflurane preconditioning confers cardioprotection by activation of ALDH2. *PLoS One* 2013; **8**: e52469 [PMID: 23468836 DOI: 10.1371/journal.pone.0052469]

64   **Churchill EN,** Disatnik MH, Mochly-Rosen D. Time-dependent and ethanol- induced cardiac protection from ischemia mediated by mitochondrial translocation of epsilon PKC and activation of aldehyde dehydrogenase 2. *J Mol Cell Cardiol* 2009**;** **46**: 278-284

65   **Budas GR,** Disatnik MH, Chen CH, Mochly-Rosen D. Activation of aldehyde dehydrogenase 2 (ALDH2) confers cardioprotection in protein kinase C epsilon knockout mice. *J Mol Cell Cardiol* 2010; **48**: 757-764

66   **Duranski MR**, Greer JJ, Dejam A, Jaganmohan S, Hogg N, Langston W, Patel RP, Yet SF, Wang X, Kevil CG, Gladwin MT, Lefer DJ. Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. *J Clin Invest* 2005; **115**: 1232-1240 [PMID: 15841216 DOI: 10.1172/JCI22493]

67   **Chiari PC**, Bienengraeber MW, Weihrauch D, Krolikowski JG, Kersten JR, Warltier DC, Pagel PS. Role of endothelial nitric oxide synthase as a trigger and mediator of isoflurane-induced delayed preconditioning in rabbit myocardium. *Anesthesiology* 2005; **103**: 74-83 [PMID: 15983459]

68   **Amour J**, Brzezinska AK, Weihrauch D, Billstrom AR, Zielonka J, Krolikowski JG, Bienengraeber MW, Warltier DC, Pratt PF Jr, Kersten JR. Role of heat shock protein 90 and endothelial nitric oxide synthase during early anesthetic and ischemic preconditioning. *Anesthesiology* 2009; **110**: 317-325 [PMID: 19194158 DOI: 10.1097/ALN.0b013e3181942cb4]

69   **Lundberg JO**, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov* 2008; **7**: 156-167 [PMID: 18167491 DOI: 10.1038/nrd2466]

70   **Oelze M**, Knorr M, Schell R, Kamuf J, Pautz A, Art J, Wenzel P, Münzel T, Kleinert H, Daiber A. Regulation of human mitochondrial aldehyde dehydrogenase (ALDH-2) activity by electrophiles in vitro. *J Biol Chem* 2011; **286**: 8893-8900 [DOI: 10.1074/jbc.M110.190017]

71   **Arif S**, Borgognone A, Lin EL, O'Sullivan AG, Sharma V, Drury NE, Menon A, Nightingale P, Mascaro J, Bonser RS, Horowitz JD, Feelisch M, Frenneaux MP, Madhani M. Role of aldehyde dehydrogenase in hypoxic vasodilator effects of nitrite in rats and humans. *Br J Pharmacol* 2015; **172**: 3341-3352 [PMID: 25754766 DOI: 10.1111/bph.13122]

72   **Rassaf T**, Totzeck M, Hendgen-Cotta UB, Shiva S, Heusch G, Kelm M. Circulating nitrite contributes to cardioprotection by remote ischemic preconditioning. *Circ Res* 2014; **114**: 1601-1610 [PMID: 24643960 DOI: 10.1161/CIRCRESAHA.114.303822]

73   **Ormerod JO**, Evans JD, Contractor H, Beretta M, Arif S, Fernandez BO, Feelisch M, Mayer B, Kharbanda RK, Frenneaux MP, Ashrafian H. Human Second Window Pre-Conditioning and Post-Conditioning by Nitrite Is Influenced by a Common Polymorphism in Mitochondrial Aldehyde Dehydrogenase. *JACC Basic Transl Sci* 2017; **2**: 13-21 [PMID: 28280793 DOI: 10.1016/j.jacbts.2016.11.006]

74   **Gong D**, Zhang Y, Zhang H, Gu H, Jiang Q, Hu S. Aldehyde dehydrogenase-2 activation during cardioplegic arrest enhances the cardioprotection against myocardial ischemia-reperfusion injury. *Cardiovasc Toxicol* 2012; **12**: 350-358 [PMID: 22814936 DOI: 10.1007/s12012-012-9179-6]

75   **Adam R**, Delvart V, Karam V, Ducerf C, Navarro F, Letoublon C, Belghiti J, Pezet D, Castaing D, Le Treut YP, Gugenheim J, Bachellier P, Pirenne J, Muiesan P; ELTR contributing centres, the European Liver, Intestine Transplant Association (ELITA). Compared efficacy of preservation solutions in liver transplantation: a long-term graft outcome study from the European Liver Transplant Registry. *Am J Transplant* 2015; **15**: 395-406 [PMID: 25612492 DOI: 10.1111/ajt.13060]

76   **Arnau Panisello-Rosello**, Alexandre Lopez, Carlos Castro, Sravan Balloji, Emma Folch-Puy, Anabela Rolo, Carlos Palmeira, René Adam and Joan Roselló-Catafau. Involvement of aldehyde

dehydrogenase 2 (ALDH2) in fatty liver graft protection mechanisms against cold ischemic injury. Poster session presented at: American Transplant Congress 2018; June 2-6; Seattle, Washington, USA

77   **Marino A,** Levi R. Salvaging the ischemic heart: Gi-coupled receptors in mast cells activate a PKC/ALDH2 pathway providing anti-RAS cardioprotection. *Curr Med Chem* 2018 [DOI: 10.2174/0 929867325666180214115127]

78   **Zhang T**, Zhao Q, Ye F, Huang CY, Chen WM, Huang WQ. Alda-1, an ALDH2 activator, protects against hepatic ischemia/ reperfusion injury in rats via inhibition of oxidative stress. *Free Radic Res* 2018; **52**: 629-638 [PMID: 29589772 DOI: 10.1080/107 15762.2018.1459042]

MINIREVIEWS

Multiparametric analysis of colorectal cancer immune responses

Julia KH Leman, Sarah K Sandford, Janet L Rhodes, Roslyn A Kemp

Julia KH Leman, Sarah K Sandford, Janet L Rhodes, Roslyn A Kemp, Department of Microbiology and Immunology, University of Otago, Dunedin 9010, New Zealand

ORCID number: Julia KH Leman (0000-0003-1966-0394); Sarah K Sandford (0000-0002-2901-4516); Janet L Rhodes (0000-0002-6407-2770); Roslyn A Kemp (0000-0001-9092-1150).

Abstract

Colorectal cancer (CRC) is a heterogeneous disease, with a diverse and plastic immune cell infiltrate. These immune cells play an important role in regulating tumour growth - progression or elimination. Some populations of cells have a strong correlation with disease-free survival, making them useful prognostic markers. In particular, the infiltrate of CD3^{+} and CD8^{+} T cells into CRC tumours has been validated worldwide as a valuable indicator of patient prognosis. However, the heterogeneity of the immune response, both between patients with tumours of different molecular subtypes, and within the tumour itself, necessitates the use of multiparametric analysis in the investigation of tumour-specific immune responses. This review will outline the multiparametric analysis techniques that have been developed and applied to studying the role of immune cells in the tumour, with a focus on colorectal cancer. Because much of the data in this disease relates to T cell subsets and heterogeneity, we have used T cell populations as examples throughout. Flow and mass cytometry give a detailed representation of the cells within the tumour in a single-cell suspension on a per-cell basis. Imaging technologies, such as imaging mass cytometry, are used to investigate increasing numbers of markers whilst retaining the spatial and structural information of the tumour section and the infiltrating immune cells. Together, the analyses of multiple immune parameters can provide valuable information to guide clinical decision-making in CRC.

Key words: Colorectal cancer; Flow cytometry; Immune cells; Multiparametric analysis; Immunohistochemistry; Mass cytometry; Microscopy

Core tip: Colorectal cancer (CRC) is a heterogeneous disease. The immune response to CRC is highly variable,

clinically relevant, and as yet poorly characterised. Clinical management of this disease is still relatively uniform, and does not yet accommodate the influence of immune response on patient outcomes. Multiparametric analysis of CRC is the best approach to describe the immune response. Improved understanding of immune responses to CRC will guide patient management, improved survival, and identify new potential therapeutic biomarkers. This review summarises currently available modes of multiparametric analysis, their advantages and drawbacks, and their clinical relevance.

INTRODUCTION

The immune response has long been established as an important factor in determining the outcome of people with cancer. Studies using immunodeficient animal models of cancer demonstrated that tumour growth was enhanced in the absence of an immune system[1,2]. This phenomenon was later observed in humans undergoing immunosuppressive therapies[3]. Immune based therapies have been developed over the last 30 years, including immune cell transfer, immune priming and inhibition of immune checkpoints (reviewed in[4]), with much success in many types of cancer. These immunotherapies primarily target the adaptive immune system, which coordinates a tumour-specific response. However, despite the fact that a high T cell infiltrate is a better indicator of patient survival than traditional histological based staging methods[5], the immune response in colorectal cancer (CRC) has not yet been reliably and effectively harnessed to treat patients of all stages and types of disease[6,7].

CRC is a highly heterogeneous disease[8] and the immune response in cancer is equally complex. Both factors complicate research into the development of immune therapies for CRC, as well as improving treatment decisions for clinicians. Analyses of tumour-immune cell interactions require the use of a range of parameters to avoid oversimplification, and ensure the disease is captured in its entirety. Technological advances in cytometry and cytometric imaging have exponentially broadened the scope of immunological study. Use of the light and ionic mass spectrums have enabled researchers to label up to 40 parameters, both cell surface and intracellular, to interrogate the immune system on a cell-by-cell basis[9-13]. Analysis of many parameters on each cell is essential to understand the functions of the many cell groups which exist in a heterogeneous population.

In this review, we describe the need for multiparametric analysis to investigate the immune response in CRC, with a focus on how this can improve clinical outcomes. Multiparametric tools such as polychromatic flow cytometry, mass cytometry and imaging technologies, and their use in these studies, will be reviewed. We will argue that use of these techniques is essential to obtain meaningful immune data in the context of fundamental CRC research and that they can be included into ongoing and future clinical trials to improve development of new therapies.

HETEROGENEITY OF CRC AND THE EFFECT ON THE LOCAL IMMUNE RESPONSE

CRC is a heterogeneous disease with different molecular subtypes[14-16]. Patients with microsatellite instability (MSI)^high tumours tend to respond better to targeted immunotherapy and have improved disease-free survival than MSI^negative patients[17]. A meta-analysis of 18 CRC datasets revealed that tumours could be subtyped into four groups: MSI immune, canonical, metabolic and mesenchymal[18]. These subtypes group tumours by mutational status and chromosomal instability, somatic copy number variation, immune infiltration and metabolic regulation, and could be correlated to staging and survival outcomes[18].

The immune cells infiltrating the tumour in CRC are heterogeneous[19]. Macrophages, dendritic cells, neutrophils and lymphocytes, such as T cells, have all been studied in the context of colorectal tumours, and different populations of these cells control tumour progression or rejection, ultimately affecting overall outcome for the patient[20]. Table 1 summarises the conflicting data between infiltrating immune cell populations and the effect on CRC patient outcome. However, heterogeneity exists within these subsets, which can alter their effect in the tumour. For instance, the study of different neutrophil populations has led to conflicting evidence about their role in colorectal tumours[21-23]. Two studies used immunohistochemistry (IHC) to count myeloperoxidase (MPO)^+ cells present in the tumour. Roncucci *et al*[22] showed that an MPO^+ cell infiltrate correlated with colonic inflammation, and that these cells were a CRC risk indicator. Droeser *et al*[21] later showed that a high MPO^+ cell infiltrate was associated with better prognosis, and that MPO was mostly expressed by CD15^+ cells. CD15 is a granulocyte marker, which is also expressed at low levels on monocytes[24]. Many studies have highlighted the diversity of T cell infiltrates in CRC, and have linked these subpopulations with patient outcome[25-31].

However, the immune response is not independent of the tumour microenvironment. Tumour type and associated molecular profile are associated with different levels of immune infiltrate[18]. The MSI status of a

Table 1 Relationship between different immune cell subsets and colorectal cancer prognosis

Type of immune cell	Canonical function	Role in colorectal cancer	Ref.
CD8^{+} T cells	Production of cytokines (including IFN-γ) and cytotoxic molecules	High numbers of CD8^{+} T cells in the invasive margin correlate with favourable prognosis.	[56]
RORγT^{+}IL-17^{+} T cells	Production of IL-17, which recruits inflammatory cells such as neutrophils	Associated with poor prognosis, especially in combination with low levels of Th1 cells (Tbet[+]IRF1^{+} IL12Rβ2^{+}STAT4^{+})	[31]
Regulatory T (Treg) cells	Regulation and suppression of effector T cell responses, production of IL-10 and TGF-β.	High density of CD3^{+}FOXP3^{+} Tregs associated with improved disease-free survival.	[11]
Effector Treg cells	Regulation of T cell responses, production of cytokines	CD3^{+}FOXP3^{+}Blimp-1^{+} associated with increased disease-free survival.	[72]
Macrophages	Phagocytic cells with pro- or anti-inflammatory properties, recruit T cells, neutrophils	Associated with favourable prognosis at the invasive margin.	[51]
Neutrophils	Phagocytosis of infected, damaged or dying cells, including tumours	Conflicting results, but a high ratio of neutrophils: CD8^{+}T cells associated with poor prognosis.	[21-23,86]
Dendritic cells	Antigen presenting cells	Mature tumour-infiltrating (S100^{+}CD83^{+}) dendritic cells associated with improved prognosis.	[87]

Blimp-1: B lymphocyte-induced maturation protein 1; FOXP3: Forkhead box protein 3; IFN-γ: Interferon-gamma; TGF-β: Transforming growth factor-beta; Treg: Regulatory T cell.

tumour has a strong effect on the number of infiltrating CD3^{+} lymphocytes: MSI^{high} tumours are associated with higher levels of CD8^{+} T cells within the tumour, and with higher levels of infiltrating forkhead box protein (FOXP3)[+] regulatory T (Treg) cells[32,33].

Soluble mediators produced by tumour cells can have inflammatory or immunomodulatory effects, which dictate the function of both tumour and immune cells. Tumours produce angiogenic factors that promote growth, but which can also inhibit activity of the immune infiltrate. Vascular endothelial growth factor (VEGF) is a proangiogenic factor produced by tumour cells[34], and which has immunomodulatory effects on macrophages, T cells and dendritic cells (reviewed in[35]). VEGF led to the maturation of CD34^{+} haematopoietic stem cells into dendritic cells[36], and also induced conversion of immature dendritic cells into endothelial-like cells, which may contribute to neovascularisation[37].

The cytokine environment has a significant impact on immune cell function and activity. In a Rag^{-/-} mouse model of mucosal inflammatory hypoxia, Treg formation was induced through induction of FoxP3 expression in a transforming growth factor (TGF)-β dependent manner[38]. TGF-β also has a direct pro-oncogenic function in cancer. As well as promoting invasion and metastasis of tumour cells, TGF-β inhibits the anti-tumour immune response by modulating proliferation, function and differentiation of both myeloid and lymphocyte populations (reviewed in[39]). In colorectal cancer patients with MSI[high] tumours, mutations in the TGF-β receptor (TGF-β1 RII) were associated with a higher rate of disease free survival and a lower risk of recurrence than MSI[high] tumours without TGF-β1 RII mutations[40].

IMMUNE CELL PLASTICITY

Immune cells change phenotype and function in response to changes in the cytokine environment

or to other stimuli. T cells differentiate into different subtypes when activated by different pathogens, and plasticity between these subtypes has been shown in many models (reviewed in[41]). In a study of T cells from healthy human blood, a high degree of heterogeneity was found within canonical T cell subsets[42]. T cells with an intermediate phenotype between different subsets indicated plasticity even in healthy individuals[42].

The tumour microenvironment induces changes in the phenotype of many types of immune cells. The repolarisation of suppressive Treg cells within the tumour was shown with the discovery of a FOXP3^{+} population expressing canonical inflammatory markers interleukin (IL)-17 and RORγt, in patients with CRC[43]. Expression of IL-17 by tumour infiltrating lymphocytes was associated with poor survival in CRC patients[31]. These data show that the tumour can manipulate T cells from an immunoregulatory state to an inflammatory pro-tumour state. Both macrophages and neutrophils have been shown to change phenotype and function within the CRC tumour microenvironment[44,45]. For example, soluble factors in the tumour microenvironment, such as TGF-β and IL-13, can drive macrophages into a suppressive phenotype, which promotes tumour progression[46]. In patients with CRC, tumour-associated macrophages (TAMs) had distinct phenotypes to those found in adjacent non-tumour bowel. An increase in populations resembling both pro- and anti-inflammatory macrophages was found within the tumour[19].

TUMOUR-IMMUNE CELL INTERACTIONS-SPATIAL HETEROGENEITY

Tumour-immune cell interactions are spatially heterogeneous, and different infiltrates in different parts of the tumour are associated with different outcomes. As early as 2009, it was shown that the infiltrate of T cells in the invasive margin of CRC tumours was more

predictive of patient outcome than T cells in the centre of the tumour[47]. Similarly, a high frequency of CD3^{+} T cells < 10 μm from metastatic liver CRC tumours correlated with improved overall survival[48]. A comprehensive study of the infiltrating immune cells over the course of tumour progression showed that T cell presence in the tumour decreased over time, whereas the number of B cells and innate immune cells such as macrophages and neutrophils increased with tumour progression[49]. This study also showed that the immune cell populations were distinct between the tumour core and the invasive margin. For instance, in the tumour core, the B cells were closely correlated with the T cell network, despite being present in low numbers[49]. In stage I-II CRC, a low infiltration of neutrophils in the invasive margin was associated with poor patient prognosis[50]. The presence of macrophages at the invasive margin of colorectal tumours was correlated with improved prognosis, but not their presence overall, highlighting the importance of retaining spatial information where possible[51].

Interactions between immune cells also have prognostic power. Co-localisation of CD8^{+} T cells and CD66b^{+} neutrophils predicted favourable prognosis in CRC patients[25]. Functional analyses of these cell groups showed that neutrophils from the tumour activated T cells to produce interferon-gamma (IFN-γ). The addition of functional markers into imaging analysis would have increased the power of this study[52].

TOOLS FOR MULTIPARAMETRIC ANALYSIS

Flow cytometry
Flow cytometry is a technique which enables characterisation of cells on a per-cell basis. Cell surface and intracellular proteins are labelled using epitope-specific antibodies conjugated to specific fluorophores[12,53]. Fluorescence-activated cell sorting (FACS) is a related technique, which uses flow to sort viable cells based on the expression of markers which have been tagged. This is a useful tool if further *in vitro* assays are required to investigate phenotype and function of particular cell types.

Often limited by spectral overlap and spread of the fluorophores, and low- or co-expressed antigens, multiparametric flow cytometry using more than 12 markers can require a high level of technical skill and appropriate instrumentation. By rearranging detectors and applying appropriate controls, a 17-colour panel was developed and used to successfully investigate multiple markers on T cell populations[12]. This study was a method development study that highlighted heterogeneity in pre-defined T cell subsets. The development of the bright Brilliant Violet dyes greatly extended the limits of markers which could be analysed in a single panel[54,55]. Further developments in fluorophore chemistry and use of a 50-detector system have resulted in the development of a 27-colour panel

for flow cytometry[55].

Flow cytometry has been an essential tool for cancer research. Immunohistological staining showed that more T cells in colorectal tumours correlated with better prognosis, but a causal effect on survival was elucidated using flow cytometry[56,57]. Flow cytometry has been used successfully to identify multiple subsets of immune cells in the context of colorectal cancer[27,30].

Studies using flow cytometry to investigate the role of Tregs in CRC have produced conflicting results. A high infiltrate of Tregs has been associated with negative, positive, or no effect on patient outcome (reviewed in[58]). The heterogeneity and plasticity of Treg populations may explain these conflicting results. A study which defined Tregs as CD4^{+}FOXP3^{+}CD25^{+}CD127^{lo}, using flow cytometry, found that circulating Tregs were reduced in patients with metastatic CRC who had been treated with a VEGF receptor blocker[59]. A study which used flow cytometry to investigate multiple parameters of Tregs in CRC patients revealed a population of highly immunosuppressive CD4^{+}FOXP3^{-} Treg-like cells that were enriched in the tumour, compared to adjacent colon or blood[60]. Cells in this population expressed Helios, CD39, cytotoxic T-lymphocyte antigen (CTLA-4), and latency-associated peptide (LAP). They also produced IL-10 and TGF-β, and were 50 times more suppressive than CD4^{+}FOXP3^{+} cells[60]. The heterogeneity of the Treg infiltrate necessitates the inclusion of a wide range of markers to understand their role in disease progression.

Flow cytometry has also been used to examine T cell dysfunction in CRC patients. In a study comparing immune cell phenotypes from T cells infiltrating the tumour and non-tumour bowel from matched CRC patients, a higher proportion of cells in the tumour than bowel expressed "exhaustion" markers, including programmed death ligand 1 (PD-1), LAG-3, CTLA-4 and TIM-3[30,61,62]. In a separate study of tumour draining lymph nodes, CD8^{+} T cells that expressed the activation marker PD-1 were functional in the tumour-free lymph node, but could not produce cytokines in the tumour-draining lymph node[63].

Mass cytometry
The challenges with spectral overlap and compensation experienced by users of flow cytometry have been addressed with the advent of mass cytometry. This technology shares the basic principle of flow cytometry, except monoclonal antibodies are labelled with heavy metal isotopes instead of fluorophores[64]. After cells are labelled, they are nebulised and the isotopes are measured cell-by-cell using mass spectrometry[64]. The use of heavy metal isotopes eliminates spectral overlap, therefore, the number of parameters that can be incorporated is much greater. While the technique is used for 40 parameters currently, the availability of heavy metal ions and lack of spectral overlap mean that this technology can theoretically be used to examine between 70 and 100 markers per cell[65].

The analysis of up to 40 markers gives much better

resolution as to the functional and phenotypic properties of the cells of interest. Analysis of heterogeneous cell populations requires the measurement of as many markers as possible, so that the full complexity of the immune cell-tumour interactions can be understood. Mass cytometry was used to develop a detailed model of haematopoietic development in human bone marrow samples and showed functional responses to drug interventions differed across immune cell subtypes[9]. This was one the first papers to use this technique to show the complexity of immune cell development and signalling[9].

Recently, researchers have used mass cytometry to begin studying the complex dyanmics of tumour-immune cell interactions. Spitzer *et al*[13] demonstrated that the success of immunotherapy in a mouse model required a systemic and diverse immune response. Analysis of the immune infiltrate of bone marrow, blood, tumour, spleen and lymph nodes showed that tumour rejection after immunotherapy was dependent on a system-wide response. The breadth and depth of this study revealed factors essential for immune-mediated tumour rejection in the context of immunotherapy which have until now been overlooked. Mass cytometry has since been used to develop an "immune atlas" of immune cell profiles during the development of lung adenocarcinomas[66]. The use of high dimensional single cell analysis of the early development of lung tumours highlighted modulation of innate cells as an avenue for early therapy.

A caveat of incorporating many parameters is that an analytical framework is needed to visualise the high-dimensional output, and compare datasets within the research community[67]. The tools most useful for multiparametric dataset analysis and visualisation were recently reviewed by Kimball *et al*[68].

IMAGING TECHNIQUES

The most important advantage of imaging techniques over mass cytometry and flow cytometry is that spatial and geographical information is retained. The imaging techniques described below have been used to determine that the distribution of immune cells, in relation to other immune cells and cancer cells, can not only improve the ability of clinicians to prognosticate patients with CRC but also reveal which patients will benefit from further treatment.

Immunohistochemistry
Lymphocyte infiltration related to CRC patient prognosis was established *via* histopathology in 1978[69], and IHC has been used in the last 40 years to look at the type, frequency and location of immune cells in colorectal cancer tumours. Galon *et al*[56] showed in 2006 that higher lymphocytic cell infiltrate in two tumour regions (invasive margin and centre of the tumour) correlated with improved disease-free and overall survival in CRC patients. The T cell infiltrate was a better

predictor of patient disease-free survival than current histopathological staging methods used in the clinic. These findings have helped define patient prognosis, better identify high-risk patients, and improve the quality of life by predicting patients who would benefit from further therapy. There have recently been new developments in highly multiplexed tissue imaging. For a comprehensive description of some of the following techniques, see[70].

Immunofluoresence microscopy
Immunofluorescence, using confocal microscopy to look at several markers simultaneously, provides an advantage over standard IHC. Secondary antibodies conjugated to a fluorophore are added to primary antibody-labelled tissue sections. Up to four fluorophores can typically be detected on a confocal microscope. This technique has been used to increase understanding of the immune infiltrate in colorectal tumours and how different cell subsets relate to prognosis, for example, neutrophil and CD8^{+} T cell interplay has been shown to improve survival in CRC patients[52]. Another example is the validation of the Immunoscore[5] in a New Zealand cohort of stage II CRC patients[71]. Importantly, it was found that analysis of additional markers (to identify Tregs and effector Tregs) provided means to identify patients with the highest risk of recurrent disease, meaning that these patients can be referred for further treatment to improve their prognosis.

The measurement of more than four parameters simultaneously using immunofluorescence has required a change in technology to include iterative staining protocols. An example of this technology is the Opal[TM] method, developed for fluorescent IHC in formalin-fixed paraffin-embedded (FFPE) tissue of up to seven markers. Using this platform, the tissue sample is stained in cycles with primary antibodies, secondary antibodies conjugated to HRP, and fluorophores which undergo a tyramide signal amplification reaction to covalently bond to the epitope. The antibodies are then removed without disrupting the fluorescence signals, which are measured by multispectral imaging[72]. This multiparameter imaging approach has been used in CRC research to show that TGF-β signalling proteins were increased in cancer tissues compared to normal tissues, and several proteins correlated with improved overall survival[73]. Further, spatial distribution of cytotoxic T cells in proximity to cancer cells correlated with increased overall patient survival in a study of pancreatic cancer patients[74], increased T cells at the tumour border improved overall survival in patients with CRC liver metastases[48], and Treg and CD8^{+} T cell proximity to cancer cells in non-small cell lung cancer correlate with worse and better lung cancer survival, respectively[75].

Mass cytometry-based imaging methods
Mass cytometry-based imaging methods, such as imaging mass cytometry (IMC[76]) and multiplex ion

beam imaging (MIBI[77]) can overcome many difficulties seen with immunofluorescence and allow the detection of up to 40 markers simultaneously using rare earth metal isotopes. The tissue is both ablated by a laser (IMC) or ion beam (MIBI) and carried by a stream of inert gas to a mass cytometer. The metal isotopes detected are mapped to the location of each spot to create an image of the target proteins throughout the tissue of interest[70,77,78]. So far, there are limited studies using these techniques and none in the field of CRC. Giesen *et al*[76] used IMC and Angelo *et al*[78] used MIBI on human breast cancer samples. These studies highlight the heterogeneity of tumours and why it is important to look at many parameters together. Because these techniques greatly increase the number of markers that can be looked at simultaneously, it has the potential to significantly expand our understanding of the immune response to CRC. Knowledge of a beneficial or disadvantageous immune infiltrate will enable better prognosis prediction and more personalised treatment.

Digital spatial profiling technology

Digital spatial profiling is a novel platform developed by NanoString Technologies. It combines standard immunofluorescence techniques with digital optical barcoding technology to detect and map up to 800 protein and RNA targets in FFPE samples without destroying the tissue[79]. There are few studies using this technology thus far; however, it has the potential to further increase the volume of data that can be obtained from samples with the inclusion of RNA detection.

MOLECULAR METHODS

Several molecular methods are available to study the immune infiltrate at the genetic, chromatin or transcriptional level, but these methods are outside the scope of this review. Briefly, gene expression analyses using qPCR can show functional differences in the immune infiltrate, but cell-by-cell gene expression techniques are still under development for widespread use. Assay for transposase-accessible chromatin sequencing (ATAC-seq) is used to investigate the chromatin structure of mammalian cells to show the regulation of the chromosomes, which in turn indicates the phenotype and function of a cell, and has been used to distinguish T cell activation from dysfunction[80,81]. ATAC-seq has also been developed to use on a cell-by-cell basis, for heterogeneous populations[82].

CLINICAL PERSPECTIVES

Oncologists and surgeons encounter immunologic, genetic, molecular, and cellular variables involved in CRC patient prognosis. Critical appraisal of the effect of each complex variable is not practical for the clinical specialist also charged with keeping abreast of their own field. This glut of information contributes to slow clinical uptake of promising developments. In particular, advances in the immune response to CRC are complex, often conflicting, and of quality that is difficult for the uninitiated to assess. There is therefore a need not only for detailed analysis of the immune response to CRC, but also a pathway for communicating this information to clinical practice.

The ideal multiparametric analysis of CRC tissue in the clinical setting would more accurately guide postoperative patient management. Chemotherapy is currently excluded from patient treatment when the mortality and morbidity risks inherent with treatment are thought to outweigh the risk of CRC recurrence. But current prognostic techniques are failing up to 25% of patients with apparently "low risk" CRC who experience recurrence despite their good American Joint Committee on Cancer (AJCC) prognosis. These patients often do not receive chemotherapy but with clinical hindsight would benefit from treatment. The current focus of the Immunoscore is improving patient selection for chemotherapy. Gold standard chemotherapy is multiagent[83,84]; however, components are often poorly tolerated and occasionally rationalised to single agent treatment. Future research should include large cohort studies that correlate immune infiltrate with response to chemotherapeutic agents, both single and multiagent. This could further personalise patient management to allow exclusion of risky chemotherapeutic components where appropriate.

CRC tissue banks are ideally suited to prognostic research. Retrospective banks eliminate the usual lag between tissue analysis and patient follow up, enabling immediate correlation of immune infiltrate with long term patient outcome. However, tissue is frequently stored as FFPE, as this is the current histopathological tissue preparation. Not all modes of multiparametric analysis are compatible with FFPE prepared tissue, but most methods of storage have a concordant mode of multiparametric analysis. It can be argued that there is an ethical obligation (with appropriate consent) to donor patients and families in any use of donated tissue; the researcher should collect as much information as possible from as small a sample of tissue as feasible. Multiparametric analysis facilitates this.

Clinical cohort studies have explored crude assessments of inflammation in the context of CRC, such as the neutrophil lymphocyte ratio, with indeterminate results[85-87]. This demonstrates that the importance of immune response to CRC is gradually infiltrating the clinical lexicon. The Immunoscore has far more definitive results[5] but has yet to enter routine clinical practice. However, both these scores are rudimentary quantification of immune cells, and do not reflect the heterogeneity and plasticity of the immune system. Multiparametric analysis addresses this, but adds yet more complexity to a field already overwhelming to the average clinical specialist. Encouraging clinical uptake
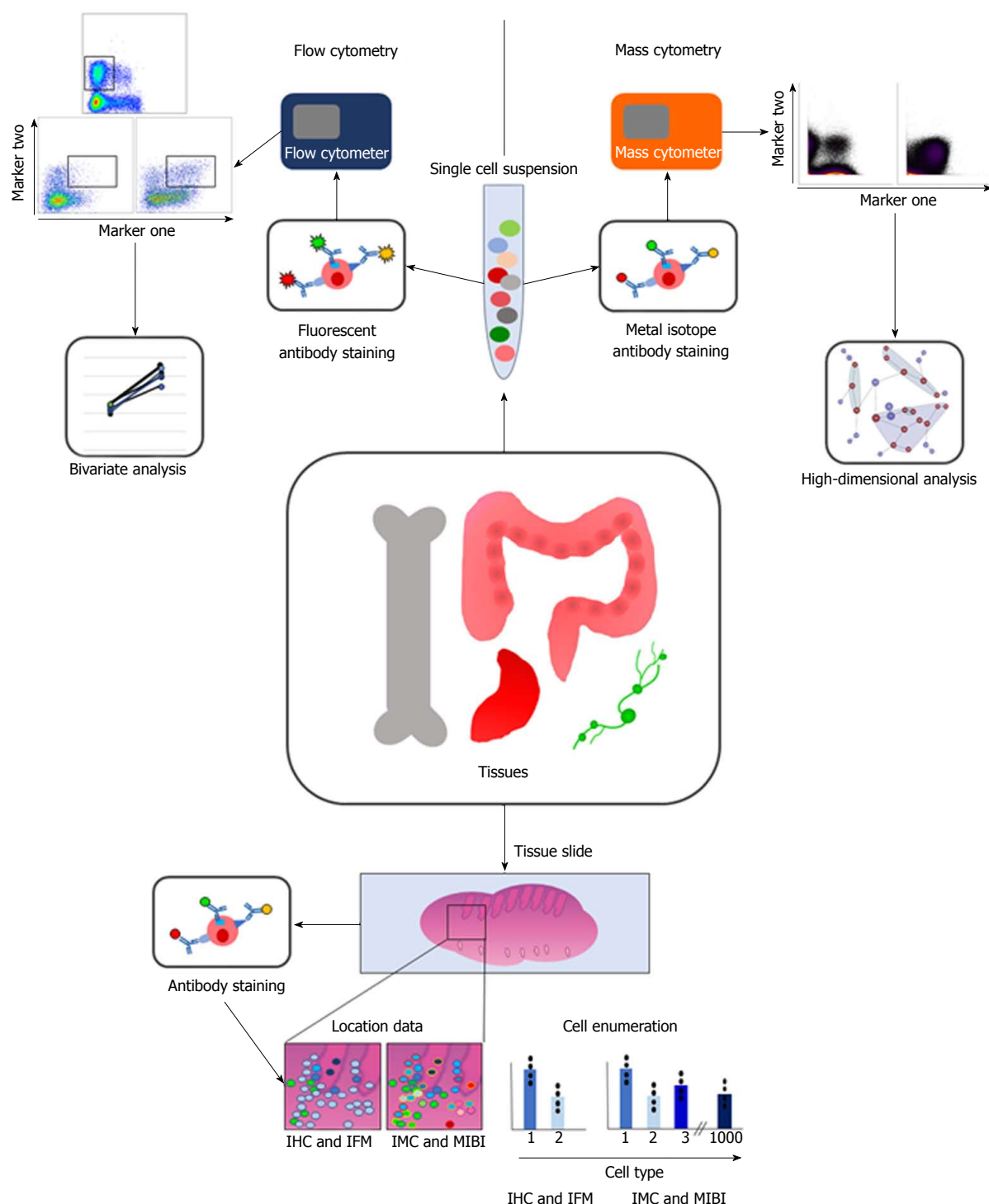
Figure 1 Diagram summarising the methodology and analysis techniques involved in multiparametric analysis of immune cells. Briefly, tissues of interest are taken and either dissociated into a single cell suspension or mounted onto slides. Cells in suspension are stained with fluorescently labelled antibodies for flow cytometry, and then acquired using a flow cytometer. This data is often presented as populations positive or negative for 2 markers. For mass cytometry, cells are stained with antibodies labelled with heavy metal isotopes, and then acquired using a mass cytometer. The high dimensionality of this data means that clustering analyses are preferred to analyse this data. For imaging techniques, the tissue slides are also labelled with antibodies, and can be imaged using IHC, IFM, IMC or MIBI. Imaging techniques enable location of immune cells *in situ*, as well as enumeration of cell types. IMC and MIBI use more parameters, meaning more cell types can be distinguished, and clustering algorithms can be used on this data too, and related back to the geographical location of each cell. IHC: Immunohistochemistry; IFM: Immunofluorescent microscopy; IMC: Imaging mass cytometry; MIBI: Multiplex ion beam imaging.

of new biomarkers will require evolution of existing communication between immunologists and clinicians. Future multidisciplinary cancer management meetings may require incorporation of immunologists, geneticists, and molecular pathologists (Figure 1).

CONCLUSION

The complexity and heterogeneity of tumour-immune cell interactions necessitate the use of multiparametric analyses. CRC tumours are highly heterogeneous, and

have correspondingly heterogeneous immune infiltrates. The immune cells in a single tumour are also dynamic and varied, and interact with each other and the tumour cells to affect patient prognosis, both positively and negatively. The spatial distribution of these cells can also play a role in the clinical outcome of a CRC patient.

The integration of single cell analysis, functional assays and imaging techniques can be applied in combination to better understand the role of the immune system in CRC. It should be noted that the techniques presented here are limited in that they are only qualitative, and do not always provide quantitative data. Current techniques are able to quantify and describe in detail different immune populations, but extrapolating the effects of these populations on patient outcome is limited to association studies, which require large cohorts. The next step will be combining phenotypic characterisation with functional and interaction analysis, to better understand the mechanisms through which immune cell subsets interact with the tumour *in situ*.

REFERENCES

1 **Stutman O**. Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. *Science* 1974; **183**: 534-536 [PMID: 4588620 DOI: 10.1126/science.183.4124.534]

2 **Shankaran V**, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, Schreiber RD. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* 2001; **410**: 1107-1111 [PMID: 11323675 DOI: 10.1038/35074122]

3 **Penn I**, Starzl TE. Malignant tumors arising de novo in immunosuppressed organ transplant recipients. *Transplantation* 1972; **14**: 407-417 [PMID: 4345337 DOI: 10.1097/00007890-1972 10000-00001]

4 **Khalil DN**, Smith EL, Brentjens RJ, Wolchok JD. The future of cancer treatment: immunomodulation, CARs and combination immunotherapy. *Nat Rev Clin Oncol* 2016; **13**: 273-290 [PMID: 26977780 DOI: 10.1038/nrclinonc.2016.25]

5 **Galon J**, Pagès F, Marincola FM, Angell HK, Thurin M, Lugli A, Zlobec I, Berger A, Bifulco C, Botti G, Tatangelo F, Britten CM, Kreiter S, Chouchane L, Delrio P, Arndt H, Asslaber M, Maio M, Masucci GV, Mihm M, Vidal-Vanaclocha F, Allison JP, Gnjatic S, Hakansson L, Huber C, Singh-Jasuja H, Ottensmeier C, Zwierzina H, Laghi L, Grizzi F, Ohashi PS, Shaw PA, Clarke BA, Wouters BG, Kawakami Y, Hazama S, Okuno K, Wang E, O'Donnell-Tormey J, Lagorce C, Pawelec G, Nishimura MI, Hawkins R, Lapointe R, Lundqvist A, Khleif SN, Ogino S, Gibbs P, Waring P, Sato N, Torigoe T, Itoh K, Patel PS, Shukla SN, Palmqvist R, Nagtegaal ID, Wang Y, D'Arrigo C, Kopetz S, Sinicrope FA, Trinchieri G, Gajewski TF, Ascierto PA, Fox BA. Cancer classification using the Immunoscore: a worldwide task force. *J Transl Med* 2012; **10**: 205 [PMID: 23034130 DOI: 10.1186/1479-5876-10-205]

6 **Hodi FS**, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbé C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010; **363**: 711-723 [PMID: 20525992 DOI: 10.1056/NEJMoa1003466]

7 **Chung KY**, Gore I, Fong L, Venook A, Beck SB, Dorazio P, Criscitiello PJ, Healey DI, Huang B, Gomez-Navarro J, Saltz LB. Phase II study of the anti-cytotoxic T-lymphocyte-associated antigen 4 monoclonal antibody, tremelimumab, in patients with refractory metastatic colorectal cancer. *J Clin Oncol* 2010; **28**: 3485-3490 [PMID: 20498386 DOI: 10.1200/JCO.2010.28.3994]

8 **Jass JR**. Molecular heterogeneity of colorectal cancer: Implications for cancer control. *Surg Oncol* 2007; **16** Suppl 1: S7-S9 [PMID: 18023574 DOI: 10.1016/j.suronc.2007.10.039]

9 **Bendall SC**, Simonds EF, Qiu P, Amir el-AD, Krutzik PO, Finck R, Bruggner RV, Melamed R, Trejo A, Ornatsky OI, Balderas RS, Plevritis SK, Sachs K, Pe'er D, Tanner SD, Nolan GP. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science* 2011; **332**: 687-696 [PMID: 21551058 DOI: 10.1126/science.1198704]

10 **Irish JM**, Doxie DB. High-dimensional single-cell cancer biology. *Curr Top Microbiol Immunol* 2014; **377**: 1-21 [PMID: 24671264 DOI: 10.1007/82_2014_367]

11 **Ornatsky O**, Bandura D, Baranov V, Nitz M, Winnik MA, Tanner S. Highly multiparametric analysis by mass cytometry. *J Immunol Methods* 2010; **361**: 1-20 [PMID: 20655312 DOI: 10.1016/j.jim.2010.07.002]

12 **Perfetto SP**, Chattopadhyay PK, Roederer M. Seventeen-colour flow cytometry: unravelling the immune system. *Nat Rev Immunol* 2004; **4**: 648-655 [PMID: 15286731 DOI: 10.1038/nri1416]

13 **Spitzer MH**, Carmi Y, Reticker-Flynn NE, Kwek SS, Madhireddy D, Martins MM, Gherardini PF, Prestwood TR, Chabon J, Bendall SC, Fong L, Nolan GP, Engleman EG. Systemic Immunity Is Required for Effective Cancer Immunotherapy. *Cell* 2017; **168**: 487-502.e15 [PMID: 28111070 DOI: 10.1016/j.cell.2016.12.022]

14 **Compton CC**. Colorectal carcinoma: diagnostic, prognostic, and molecular features. *Mod Pathol* 2003; **16**: 376-388 [PMID: 12692203 DOI: 10.1097/01.MP.0000062859.46942.93]

15 **De Sousa E Melo F**, Wang X, Jansen M, Fessler E, Trinh A, de Rooij LP, de Jong JH, de Boer OJ, van Leersum R, Bijlsma MF, Rodermond H, van der Heijden M, van Noesel CJ, Tuynman JB, Dekker E, Markowetz F, Medema JP, Vermeulen L. Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. *Nat Med* 2013; **19**: 614-618 [PMID: 23584090 DOI: 10.1038/nm.3174]

16 **Lengauer C**, Kinzler KW, Vogelstein B. Genetic instability in colorectal cancers. *Nature* 1997; **386**: 623-627 [PMID: 9121588 DOI: 10.1038/386623a0]

17 **Le DT**, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, Skora AD, Luber BS, Azad NS, Laheru D, Biedrzycki B, Donehower RC, Zaheer A, Fisher GA, Crocenzi TS, Lee JJ, Duffy SM, Goldberg RM, de la Chapelle A, Koshiji M, Bhaijee F, Huebner T, Hruban RH, Wood LD, Cuka N, Pardoll DM, Papadopoulos N, Kinzler KW, Zhou S, Cornish TC, Taube JM, Anders RA, Eshleman JR, Vogelstein B, Diaz LA Jr. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med* 2015; **372**: 2509-2520 [PMID: 26028255 DOI: 10.1056/NEJMoa1500596]

18 **Guinney J**, Dienstmann R, Wang X, de Reyniès A, Schlicker A, Soneson C, Marisa L, Roepman P, Nyamundanda G, Angelino P, Bot BM, Morris JS, Simon IM, Gerster S, Fessler E, De Sousa E Melo F, Missiaglia E, Ramay H, Barras D, Homicsko K, Maru D, Manyam GC, Broom B, Boige V, Perez-Villamil B, Laderas T, Salazar R, Gray JW, Hanahan D, Tabernero J, Bernards R, Friend SH, Laurent-Puig P, Medema JP, Sadanandam A, Wessels L, Delorenzi M, Kopetz S, Vermeulen L, Tejpar S. The consensus molecular subtypes of colorectal cancer. *Nat Med* 2015; **21**: 1350-1356 [PMID: 26457759 DOI: 10.1038/nm.3967]

19 **Norton SE**, Ward-Hartstonge KA, Taylor ES, Kemp RA. Immune cell interplay in colorectal cancer prognosis. *World J Gastrointest Oncol* 2015; **7**: 221-232 [PMID: 26483876 DOI: 10.4251/wjgo.v7.i10.221]

20 **Fridman WH**, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 2012; **12**: 298-306 [PMID: 22419253 DOI: 10.1038/nrc3245]

21 **Droeser RA**, Hirt C, Eppenberger-Castori S, Zlobec I, Viehl CT, Frey DM, Nebiker CA, Rosso R, Zuber M, Amicarella F, Iezzi G, Sconocchia G, Heberer M, Lugli A, Tornillo L, Oertli D, Terracciano L, Spagnoli GC. High myeloperoxidase positive cell infiltration in colorectal cancer is an independent favorable prognostic factor. *PLoS One* 2013; **8**: e64814 [PMID: 23734221

DOI: 10.1371/journal.pone.0064814]

22   **Roncucci L**, Mora E, Mariani F, Bursi S, Pezzi A, Rossi G, Pedroni M, Luppi D, Santoro L, Monni S, Manenti A, Bertani A, Merighi A, Benatti P, Di Gregorio C, de Leon PM. Myeloperoxidase-positive cell infiltration in colorectal carcinogenesis as indicator of colorectal cancer risk. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 2291-2297 [PMID: 18768495 DOI: 10.1158/1055-9965. EPI-08-0224]

23   **Rao HL**, Chen JW, Li M, Xiao YB, Fu J, Zeng YX, Cai MY, Xie D. Increased intratumoral neutrophil in colorectal carcinomas correlates closely with malignant phenotype and predicts patients' adverse prognosis. *PLoS One* 2012; **7**: e30806 [PMID: 22295111 DOI: 10.1371/journal.pone.0030806]

24   **Gustafson MP**, Lin Y, Maas ML, Van Keulen VP, Johnston PB, Peikert T, Gastineau DA, Dietz AB. A method for identification and analysis of non-overlapping myeloid immunophenotypes in humans. *PLoS One* 2015; **10**: e0121546 [PMID: 25799053 DOI: 10.1371/journal.pone.0121546]

25   **Amicarella F**, Muraro MG, Hirt C, Cremonesi E, Padovan E, Mele V, Governa V, Han J, Huber X, Droeser RA, Zuber M, Adamina M, Bolli M, Rosso R, Lugli A, Zlobec I, Terracciano L, Tornillo L, Zajac P, Eppenberger-Castori S, Trapani F, Oertli D, Iezzi G. Dual role of tumour-infiltrating T helper 17 cells in human colorectal cancer. *Gut* 2017; **66**: 692-704 [PMID: 26719303 DOI: 10.1136/gutjnl-2015-310016]

26   **Frey O**, Reichel A, Bonhagen K, Morawietz L, Rauchhaus U, Kamradt T. Regulatory T cells control the transition from acute into chronic inflammation in glucose-6-phosphate isomerase-induced arthritis. *Ann Rheum Dis* 2010; **69**: 1511-1518 [PMID: 20498199 DOI: 10.1136/ard.2009.123422]

27   **Girardin A**, McCall J, Black MA, Edwards F, Phillips V, Taylor ES, Reeve AE, Kemp RA. Inflammatory and regulatory T cells contribute to a unique immune microenvironment in tumor tissue of colorectal cancer patients. *Int J Cancer* 2013; **132**: 1842-1850 [PMID: 23002055 DOI: 10.1002/ijc.27855]

28   **Saito T**, Nishikawa H, Wada H, Nagano Y, Sugiyama D, Atarashi K, Maeda Y, Hamaguchi M, Ohkura N, Sato E, Nagase H, Nishimura J, Yamamoto H, Takiguchi S, Tanoue T, Suda W, Morita H, Hattori M, Honda K, Mori M, Doki Y, Sakaguchi S. Two FOXP3(+)CD4(+) T cell subpopulations distinctly control the prognosis of colorectal cancers. *Nat Med* 2016; **22**: 679-684 [PMID: 27111280 DOI: 10.1038/nm.4086]

29   **Salama P**, Phillips M, Grieu F, Morris M, Zeps N, Joseph D, Platell C, Iacopetta B. Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol* 2009; **27**: 186-192 [PMID: 19064967 DOI: 10.1200/JCO.2008.18.7229]

30   **Taylor ES**, McCall JL, Girardin A, Munro FM, Black MA, Kemp RA. Functional impairment of infiltrating T cells in human colorectal cancer. *Oncoimmunology* 2016; **5**: e1234573 [PMID: 27999752 DOI: 10.1080/2162402X.2016.1234573]

31   **Tosolini M**, Kirilovsky A, Mlecnik B, Fredriksen T, Mauger S, Bindea G, Berger A, Bruneval P, Fridman WH, Pagès F, Galon J. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Res* 2011; **71**: 1263-1271 [PMID: 21303976 DOI: 10.1158/0008-5472.CAN-10-2907]

32   **Michel S**, Benner A, Tariverdian M, Wentzensen N, Hoefler P, Pommerencke T, Grabe N, von Knebel Doeberitz M, Kloor M. High density of FOXP3-positive T cells infiltrating colorectal cancers with microsatellite instability. *Br J Cancer* 2008; **99**: 1867-1873 [PMID: 18985040 DOI: 10.1038/sj.bjc.6604756]

33   **Mlecnik B**, Bindea G, Angell HK, Maby P, Angelova M, Tougeron D, Church SE, Lafontaine L, Fischer M, Fredriksen T, Sasso M, Bilocq AM, Kirilovsky A, Obenauf AC, Hamieh M, Berger A, Bruneval P, Tuech JJ, Sabourin JC, Le Pessot F, Mauillon J, Rafii A, Laurent-Puig P, Speicher MR, Trajanoski Z, Michel P, Sesboüe R, Frebourg T, Pagès F, Valge-Archer V, Latouche JB, Galon J. Integrative Analyses of Colorectal Cancer Show Immunoscore Is a Stronger Predictor of Patient Survival Than Microsatellite

Instability. *Immunity* 2016; **44**: 698-711 [PMID: 26982367 DOI: 10.1016/j.immuni.2016.02.025]

34   **Khorana AA**, Ryan CK, Cox C, Eberly S, Sahasrabudhe DM. Vascular endothelial growth factor, CD68, and epidermal growth factor receptor expression and survival in patients with Stage II and Stage III colon carcinoma: a role for the host response in prognosis. *Cancer* 2003; **97**: 960-968 [PMID: 12569594 DOI: 10.1002/cncr.11152]

35   **Li YL**, Zhao H, Ren XB. Relationship of VEGF/VEGFR with immune and cancer cells: staggering or forward? *Cancer Biol Med* 2016; **13**: 206-214 [PMID: 27458528 DOI: 10.20892/j.issn.2095-3941.2015.0070]

36   **Gabrilovich DI**, Chen HL, Girgis KR, Cunningham HT, Meny GM, Nadaf S, Kavanaugh D, Carbone DP. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat Med* 1996; **2**: 1096-1103 [PMID: 8837607 DOI: 10.1038/nm1096-1096]

37   **Fernandez Pujol B**, Lucibello FC, Zuzarte M, Lütjens P, Müller R, Havemann K. Dendritic cells derived from peripheral monocytes express endothelial markers and in the presence of angiogenic growth factors differentiate into endothelial-like cells. *Eur J Cell Biol* 2001; **80**: 99-110 [PMID: 11211940 DOI: 10.1078/0171-9335-00136]

38   **Clambey ET**, McNamee EN, Westrich JA, Glover LE, Campbell EL, Jedlicka P, de Zoeten EF, Cambier JC, Stenmark KR, Colgan SP, Eltzschig HK. Hypoxia-inducible factor-1 alpha-dependent induction of FoxP3 drives regulatory T-cell abundance and function during inflammatory hypoxia of the mucosa. *Proc Natl Acad Sci U S A* 2012; **109**: E2784-E2793 [PMID: 22988108 DOI: 10.1073/pnas.1202366109]

39   **Kubiczkova L**, Sedlarikova L, Hajek R, Sevcikova S. TGF-β - an excellent servant but a bad master. *J Transl Med* 2012; **10**: 183 [PMID: 22943793 DOI: 10.1186/1479-5876-10-183]

40   **Watanabe T**, Wu TT, Catalano PJ, Ueki T, Satriano R, Haller DG, Benson AB 3rd, Hamilton SR. Molecular predictors of survival after adjuvant chemotherapy for colon cancer. *N Engl J Med* 2001; **344**: 1196-1206 [PMID: 11309634 DOI: 10.1056/NEJM200104193441603]

41   **Murphy KM**, Stockinger B. Effector T cell plasticity: flexibility in the face of changing circumstances. *Nat Immunol* 2010; **11**: 674-680 [PMID: 20644573 DOI: 10.1038/ni.1899]

42   **Kunicki MA**, Amaya Hernandez LC, Davis KL, Bacchetta R, Roncarolo MG. Identity and Diversity of Human Peripheral Th and T Regulatory Cells Defined by Single-Cell Mass Cytometry. *J Immunol* 2018; **200**: 336-346 [PMID: 29180490 DOI: 10.4049/jimmunol.1701025]

43   **Blatner NR**, Mulcahy MF, Dennis KL, Scholtens D, Bentrem DJ, Phillips JD, Ham S, Sandall BP, Khan MW, Mahvi DM, Halverson AL, Stryker SJ, Boller AM, Singal A, Sneed RK, Sarraj B, Ansari MJ, Oft M, Iwakura Y, Zhou L, Bonertz A, Beckhove P, Gounari F, Khazaie K. Expression of RORγt marks a pathogenic regulatory T cell subset in human colon cancer. *Sci Transl Med* 2012; **4**: 164ra159 [PMID: 23241743 DOI: 10.1126/scitranslmed.3004566]

44   **Fridlender ZG**, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Worthen GS, Albelda SM. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell* 2009; **16**: 183-194 [PMID: 19732719 DOI: 10.1016/j.ccr.2009.06.017]

45   **Norton SE**, Dunn ET, McCall JL, Munro F, Kemp RA. Gut macrophage phenotype is dependent on the tumor microenvironment in colorectal cancer. *Clin Transl Immunology* 2016; **5**: e76 [PMID: 27195119 DOI: 10.1038/cti.2016.21]

46   **Zhang F**, Wang H, Wang X, Jiang G, Liu H, Zhang G, Wang H, Fang R, Bu X, Cai S, Du J. TGF-β induces M2-like macrophage polarization via SNAIL-mediated suppression of a pro-inflammatory phenotype. *Oncotarget* 2016; **7**: 52294-52306 [PMID: 27418133 DOI: 10.18632/oncotarget.10561]

47   **Laghi L**, Bianchi P, Miranda E, Balladore E, Pacetti V, Grizzi F, Allavena P, Torri V, Repici A, Santoro A, Mantovani A, Roncalli M, Malesci A. CD3+ cells at the invasive margin of deeply invading (pT3-T4) colorectal cancer and risk of post-surgical metastasis:

a longitudinal study. *Lancet Oncol* 2009; **10**: 877-884 [PMID: 19656725 DOI: 10.1016/S1470-2045(09)70186-X]

48   **Berthel A**, Zoernig I, Valous NA, Kahlert C, Klupp F, Ulrich A, Weitz J, Jaeger D, Halama N. Detailed resolution analysis reveals spatial T cell heterogeneity in the invasive margin of colorectal cancer liver metastases associated with improved survival. *Oncoimmunology* 2017; **6**: e1286436 [PMID: 28405518 DOI: 10.1080/2162402X.2017.1286436]

49   **Bindea G**, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, Angell H, Fredriksen T, Lafontaine L, Berger A, Bruneval P, Fridman WH, Becker C, Pagès F, Speicher MR, Trajanoski Z, Galon J. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity* 2013; **39**: 782-795 [PMID: 24138885 DOI: 10.1016/j.immuni.2013.10.003]

50   **Wikberg ML**, Ling A, Li X, Öberg Å, Edin S, Palmqvist R. Neutrophil infiltration is a favorable prognostic factor in early stages of colon cancer. *Hum Pathol* 2017; **68**: 193-202 [PMID: 28882699 DOI: 10.1016/j.humpath.2017.08.028]

51   **Nagorsen D**, Voigt S, Berg E, Stein H, Thiel E, Loddenkemper C. Tumor-infiltrating macrophages and dendritic cells in human colorectal cancer: relation to local regulatory T cells, systemic T-cell response against tumor-associated antigens and survival. *J Transl Med* 2007; **5**: 62 [PMID: 18047662 DOI: 10.1186/1479-5876-5-62]

52   **Governa V**, Trella E, Mele V, Tornillo L, Amicarella F, Cremonesi E, Muraro MG, Xu H, Droeser R, Däster SR, Bolli M, Rosso R, Oertli D, Eppenberger-Castori S, Terracciano LM, Iezzi G, Spagnoli GC. The Interplay Between Neutrophils and CD8+ T Cells Improves Survival in Human Colorectal Cancer. *Clin Cancer Res* 2017; **23**: 3847-3858 [PMID: 28108544 DOI: 10.1158/1078-0432.CCR-16-2047]

53   **Hulett HR**, Bonner WA, Barrett J, Herzenberg LA. Cell sorting: automated separation of mammalian cells as a function of intracellular fluorescence. *Science* 1969; **166**: 747-749 [PMID: 4898615]

54   **Chattopadhyay PK**, Gaylord B, Palmer A, Jiang N, Raven MA, Lewis G, Reuter MA, Nur-ur Rahman AK, Price DA, Betts MR, Roederer M. Brilliant violet fluorophores: a new class of ultrabright fluorescent compounds for immunofluorescence experiments. *Cytometry A* 2012; **81**: 456-466 [PMID: 22489009 DOI: 10.1002/cyto.a.22043]

55   **Ashhurst TM**, Smith AL, King NJC. High-Dimensional Fluorescence Cytometry. *Curr Protoc Immunol* 2017; **119**: 5.8.1-5.8.38 [PMID: 29091263 DOI: 10.1002/cpim.37]

56   **Galon J**, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoué F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pagès F. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006; **313**: 1960-1964 [PMID: 17008531 DOI: 10.1126/science.1129139]

57   **Reissfelder C**, Stamova S, Gossmann C, Braun M, Bonertz A, Walliczek U, Grimm M, Rahbari NN, Koch M, Saadati M, Benner A, Büchler MW, Jäger D, Halama N, Khazaie K, Weitz J, Beckhove P. Tumor-specific cytotoxic T lymphocyte activity determines colorectal cancer patient prognosis. *J Clin Invest* 2015; **125**: 739-751 [PMID: 25562322 DOI: 10.1172/JCI74894]

58   **Ward-Hartstonge KA**, Kemp RA. Regulatory T-cell heterogeneity and the cancer immune response. *Clin Transl Immunology* 2017; **6**: e154 [PMID: 28983402 DOI: 10.1038/cti.2017.43]

59   **Terme M**, Pernot S, Marcheteau E, Sandoval F, Benhamouda N, Colussi O, Dubreuil O, Carpentier AF, Tartour E, Taieb J. VEGFA-VEGFR pathway blockade inhibits tumor-induced regulatory T-cell proliferation in colorectal cancer. *Cancer Res* 2013; **73**: 539-549 [PMID: 23108136 DOI: 10.1158/0008-5472.CAN-12-2325]

60   **Scurr M**, Ladell K, Besneux M, Christian A, Hockey T, Smart K, Bridgeman H, Hargest R, Phillips S, Davies M, Price D, Gallimore A, Godkin A. Highly prevalent colorectal cancer-infiltrating LAP⁺ Foxp3⁻ T cells exhibit more potent immunosuppressive activity than Foxp3⁺ regulatory T cells. *Mucosal Immunol* 2014; **7**: 428-439 [PMID: 24064667 DOI: 10.1038/mi.2013.62]

61   **Wherry EJ**, Ha SJ, Kaech SM, Haining WN, Sarkar S, Kalia V, Subramaniam S, Blattman JN, Barber DL, Ahmed R. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 2007; **27**: 670-684 [PMID: 17950003 DOI: 10.1016/j.immuni.2007.09.006]

62   **Jin HT**, Anderson AC, Tan WG, West EE, Ha SJ, Araki K, Freeman GJ, Kuchroo VK, Ahmed R. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proc Natl Acad Sci USA* 2010; **107**: 14733-14738 [PMID: 20679213 DOI: 10.1073/pnas.1009731107]

63   **Wu X**, Zhang H, Xing Q, Cui J, Li J, Li Y, Tan Y, Wang S. PD-1(+) CD8(+) T cells are exhausted in tumours and functional in draining lymph nodes of colorectal cancer patients. *Br J Cancer* 2014; **111**: 1391-1399 [PMID: 25093496 DOI: 10.1038/bjc.2014.416]

64   **Spitzer MH**, Nolan GP. Mass Cytometry: Single Cells, Many Features. *Cell* 2016; **165**: 780-791 [PMID: 27153492 DOI: 10.1016/j.cell.2016.04.019]

65   **Bendall SC**, Nolan GP, Roederer M, Chattopadhyay PK. A deep profiler's guide to cytometry. *Trends Immunol* 2012; **33**: 323-332 [PMID: 22476049 DOI: 10.1016/j.it.2012.02.010]

66   **Lavin Y**, Kobayashi S, Leader A, Amir ED, Elefant N, Bigenwald C, Remark R, Sweeney R, Becker CD, Levine JH, Meinhof K, Chow A, Kim-Shulze S, Wolf A, Medaglia C, Li H, Rytlewski JA, Emerson RO, Solovyov A, Greenbaum BD, Sanders C, Vignali M, Beasley MB, Flores R, Gnjatic S, Pe'er D, Rahman A, Amit I, Merad M. Innate Immune Landscape in Early Lung Adenocarcinoma by Paired Single-Cell Analyses. *Cell* 2017; **169**: 750-765.e17 [PMID: 28475900 DOI: 10.1016/j.cell.2017.04.014]

67   **Spitzer MH**, Gherardini PF, Fragiadakis GK, Bhattacharya N, Yuan RT, Hotson AN, Finck R, Carmi Y, Zunder ER, Fantl WJ, Bendall SC, Engleman EG, Nolan GP. IMMUNOLOGY. An interactive reference framework for modeling a dynamic immune system. *Science* 2015; **349**: 1259425 [PMID: 26160952 DOI: 10.1126/science.1259425]

68   **Kimball AK**, Oko LM, Bullock BL, Nemenoff RA, van Dyk LF, Clambey ET. A Beginner's Guide to Analyzing and Visualizing Mass Cytometry Data. *J Immunol* 2018; **200**: 3-22 [PMID: 29255085 DOI: 10.4049/jimmunol.1701494]

69   **Watt AG**, House AK. Colonic carcinoma: a quantitative assessment of lymphocyte infiltration at the periphery of colonic tumors related to prognosis. *Cancer* 1978; **41**: 279-282 [PMID: 626936]

70   **Bodenmiller B**. Multiplexed Epitope-Based Tissue Imaging for Discovery and Healthcare Applications. *Cell Syst* 2016; **2**: 225-238 [PMID: 27135535 DOI: 10.1016/j.cels.2016.03.008]

71   **Ward-Hartstonge KA**, McCall JL, McCulloch TR, Kamps AK, Girardin A, Cretney E, Munro FM, Kemp RA. Inclusion of BLIMP-1+ effector regulatory T cells improves the Immunoscore in a cohort of New Zealand colorectal cancer patients: a pilot study. *Cancer Immunol Immunother* 2017; **66**: 515-522 [PMID: 28116480 DOI: 10.1007/s00262-016-1951-1]

72   **Stack EC**, Wang C, Roman KA, Hoyt CC. Multiplexed immunohistochemistry, imaging, and quantitation: a review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis. *Methods* 2014; **70**: 46-58 [PMID: 25242720 DOI: 10.1016/j.ymeth.2014.08.016]

73   **Yang L**, Liu Z, Tan J, Dong H, Zhang X. Multispectral imaging reveals hyper active TGF-β signaling in colorectal cancer. *Cancer Biol Ther* 2018; **19**: 105-112 [PMID: 29219668 DOI: 10.1080/15384047.2017.1395116]

74   **Carstens JL**, Correa de Sampaio P, Yang D, Barua S, Wang H, Rao A, Allison JP, LeBleu VS, Kalluri R. Spatial computation of intratumoral T cells correlates with survival of patients with pancreatic cancer. *Nat Commun* 2017; **8**: 15095 [PMID: 28447602 DOI: 10.1038/ncomms15095]

75   **Barua S**, Fang P, Sharma A, Fujimoto J, Wistuba I, Rao AUK, Lin SH. Spatial interaction of tumor cells and regulatory T cells correlates with survival in non-small cell lung cancer. *Lung Cancer* 2018; **117**: 73-79 [PMID: 29409671 DOI: 10.1016/j.lungcan.2018.01.022]

76   **Giesen C**, Wang HA, Schapiro D, Zivanovic N, Jacobs A,

Hattendorf B, Schüffler PJ, Grolimund D, Buhmann JM, Brandt S, Varga Z, Wild PJ, Günther D, Bodenmiller B. Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry. *Nat Methods* 2014; **11**: 417-422 [PMID: 24584193 DOI: 10.1038/nmeth.2869]

77   **Angelo M**, Bendall SC, Finck R, Hale MB, Hitzman C, Borowsky AD, Levenson RM, Lowe JB, Liu SD, Zhao S, Natkunam Y, Nolan GP. Multiplexed ion beam imaging of human breast tumors. *Nat Med* 2014; **20**: 436-442 [PMID: 24584119 DOI: 10.1038/nm.3488]

78   **Chang Q**, Ornatsky OI, Siddiqui I, Loboda A, Baranov VI, Hedley DW. Imaging Mass Cytometry. *Cytometry A* 2017; **91**: 160-169 [PMID: 28160444 DOI: 10.1002/cyto.a.23053]

79   **Kulkarni MM**. Digital multiplexed gene expression analysis using the NanoString nCounter system. *Curr Protoc Mol Biol* 2011; **25**: Unit25B.10 [PMID: 21472696 DOI: 10.1002/0471142727. mb25b10s94]

80   **Philip M**, Fairchild L, Sun L, Horste EL, Camara S, Shakiba M, Scott AC, Viale A, Lauer P, Merghoub T, Hellmann MD, Wolchok JD, Leslie CS, Schietinger A. Chromatin states define tumour-specific T cell dysfunction and reprogramming. *Nature* 2017; **545**: 452-456 [PMID: 28514453 DOI: 10.1038/nature22367]

81   **Buenrostro JD**, Wu B, Chang HY, Greenleaf WJ. ATAC-seq: A Method for Assaying Chromatin Accessibility Genome-Wide. *Curr Protoc Mol Biol* 2015; **109**: 21.29.1-21.29.9 [PMID: 25559105 DOI: 10.1002/0471142727.mb2129s109]

82   **Buenrostro JD**, Wu B, Litzenburger UM, Ruff D, Gonzales ML, Snyder MP, Chang HY, Greenleaf WJ. Single-cell chromatin accessibility reveals principles of regulatory variation. *Nature* 2015;

523: 486-490 [PMID: 26083756 DOI: 10.1038/nature14590]

83   **André T**, Boni C, Navarro M, Tabernero J, Hickish T, Topham C, Bonetti A, Clingan P, Bridgewater J, Rivera F, de Gramont A. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. *J Clin Oncol* 2009; **27**: 3109-3116 [PMID: 19451431 DOI: 10.1200/JCO.2008.20.6771]

84   **Tournigand C**, André T, Bonnetain F, Chibaudel B, Lledo G, Hickish T, Tabernero J, Boni C, Bachet JB, Teixeira L, de Gramont A. Adjuvant therapy with fluorouracil and oxaliplatin in stage II and elderly patients (between ages 70 and 75 years) with colon cancer: subgroup analyses of the Multicenter International Study of Oxaliplatin, Fluorouracil, and Leucovorin in the Adjuvant Treatment of Colon Cancer trial. *J Clin Oncol* 2012; **30**: 3353-3360 [PMID: 22915656 DOI: 10.1200/JCO.2012.42.5645]

85   **Guthrie GJ**, Charles KA, Roxburgh CS, Horgan PG, McMillan DC, Clarke SJ. The systemic inflammation-based neutrophil-lymphocyte ratio: experience in patients with cancer. *Crit Rev Oncol Hematol* 2013; **88**: 218-230 [PMID: 23602134 DOI: 10.1016/j.critr evonc.2013.03.010]

86   **Haram A**, Boland MR, Kelly ME, Bolger JC, Waldron RM, Kerin MJ. The prognostic value of neutrophil-to-lymphocyte ratio in colorectal cancer: A systematic review. *J Surg Oncol* 2017; **115**: 470-479 [PMID: 28105646 DOI: 10.1002/jso.24523]

87   **Gulubova MV**, Ananiev JR, Vlaykova TI, Yovchev Y, Tsoneva V, Manolova IM. Role of dendritic cells in progression and clinical outcome of colon cancer. *Int J Colorectal Dis* 2012; **27**: 159-169 [PMID: 22065108 DOI: 10.1007/s00384-011-1334-1]

ORIGINAL ARTICLE

Retrospective Study

Push *vs* pull method for endoscopic ultrasound-guided fine needle aspiration of pancreatic head lesions: Propensity score matching analysis

Mitsuru Sugimoto, Tadayuki Takagi, Rei Suzuki, Naoki Konno, Hiroyuki Asama, Yuki Sato, Hiroki Irie, Ko Watanabe, Jun Nakamura, Hitomi Kikuchi, Yuichi Waragai, Mika Takasumi, Minami Hashimoto, Yuko Hashimoto, Takuto Hikichi, Hiromasa Ohira

Mitsuru Sugimoto, Tadayuki Takagi, Rei Suzuki, Naoki Konno, Hiroyuki Asama, Yuki Sato, Hiroki Irie, Ko Watanabe, Jun Nakamura, Hitomi Kikuchi, Yuichi Waragai, Mika Takasumi, Minami Hashimoto, Hiromasa Ohira, Department of Gastroenterology, Fukushima Medical University, School of Medicine, Fukushima 960-1247, Japan

Ko Watanabe, Jun Nakamura, Hitomi Kikuchi, Minami Hashimoto, Takuto Hikichi, Department of Endoscopy, Fukushima Medical University Hospital, Fukushima 960-1247, Japan

Yuko Hashimoto, Department of Diagnostic Pathology, Fukushima Medical University, Fukushima 960-1247, Japan

ORCID number: Mitsuru Sugimoto (0000-0002-4223-613X); Tadayuki Takagi (0000-0003-0696-5973); Rei Suzuki (0000-0002-4049-0484); Naoki Konno (0000-0001-9830-4317); Hiroyuki Asama (0000-0002-0102-0404); Yuki Sato (0000-0001-8000-0972); Hiroki Irie (0000-0002-4805-6244); Ko Watanabe (0000-0003-3895-7636); Jun Nakamura (0000-0001-6006-1778); Hitomi Kikuchi (0000-0003-0583-1623); Yuichi Waragai (0000-0003-3347-8759); Mika Takasumi (0000-0002-6025-8084); Minami Hashimoto (0000-0002-5750-7182); Yuko Hashimoto (0000-0003-3435-6665); Takuto Hikichi (0000-0002-9815-1557); Hiromasa Ohira (0000-0003-4331-0634).

Author contributions: Sugimoto M designed and performed the study; Sugimoto M, Takagi T and Ohira H analyzed the data and wrote the paper; Suzuki R, Konno N, Asama H, Sato Y, Irie H, Watanabe K, Nakamura J, Kikuchi H, Waragai Y, Takasumi M, Hashimoto M and Hikichi T provided clinical advice; Hashimoto Y performed pathological diagnoses; and Hikichi T and Ohira H supervised the study.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of Fukushima Medical University.

Abstract

AIM
To evaluate the efficacy of endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) of pancreatic head cancer when pushing (push method) or pulling the echoendoscope (pull method).

METHODS
Overall, 566 pancreatic cancer patients had their first EUS-FNA between February 2001 and December 2017. Among them, 201 who underwent EUS-FNA for pancreatic head lesions were included in this study. EUS-FNA was performed by the push method in 85 patients, the pull method in 101 patients and both the push and pull methods in 15 patients. After propensity score matching (age, sex, tumor diameter, and FNA needle), 85 patients each were stratified into the push and pull groups. Patient characteristics and EUS-FNA-related factors were compared between the two groups.

RESULTS
Patient characteristics were not significantly different between the two groups. The distance to lesion was significantly longer in the push group than in the pull group (13.9 ± 4.9 mm *vs* 7.0 ± 4.9 mm, $P < 0.01$). The push method was a significant factor influencing the distance to lesion (≥ median 10 mm) ($P < 0.01$). Additionally, tumor diameter ≥ 25 mm (OR = 1.91, 95%CI: 1.02-3.58, $P = 0.043$) and the push method (OR = 1.91, 95%CI: 1.03-3.55, $P = 0.04$) were significant factors contributing to the histological diagnosis of malignancy.

CONCLUSION
The pull method shortened the distance between the endoscope and the lesion and facilitated EUS-FNA of pancreatic head cancer. The push method contributed to the histological diagnosis of pancreatic head cancer using EUS-FNA specimens.

Key words: Endoscopic ultrasound-guided fine needle aspiration; Pancreatic head; Pancreatic cancer; Push method; Pull method

Core tip: Solid pancreatic head lesions are punctured by the push or pull method. We evaluated the efficacy of endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) using the push and pull methods for pancreatic head cancer. After propensity score matching (age, sex, tumor diameter, and FNA needle), 85 patients each were stratified into the push and pull groups. Patient characteristics and EUS-FNA-related factors were compared between the two groups. The pull method shortened the distance between the endoscope and the lesion and facilitated EUS-FNA of pancreatic head cancer. The push method contributed to the histological diagnosis of pancreatic head cancer using EUS-FNA specimens.

INTRODUCTION

Endoscopic ultrasonography is superior to other imaging modalities for visualizing solid pancreatic lesions[1,2]. Endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) plays an important role in the diagnosis of pancreatic cancer. The reported diagnostic sensitivity of EUS-FNA for solid pancreatic lesions is 79%-95.0%, the specificity is 75.0%-100%, and the accuracy is 78.0%-96.0%[3-8]. The use of EUS-FNA is widespread irrespective of the type of hospital[9].

However, EUS-FNA is difficult in some situations. Regarding the location of pancreatic lesions, some reports have described the difficulty of EUS-FNA for lesions in the pancreatic head. Haba *et al*[10] reported that the pancreatic head was an independent factor affecting the accuracy of EUS-FNA for solid pancreatic lesions. Tadic *et al*[11] reported that 69% of solid pancreatic lesions with intermediate or negative cytological diagnosis by initial EUS-FNA were localized in the pancreatic head. Varadarajulu *et al*[12] reported that six pancreatic cancers with false-negative results by EUS-FNA were localized in the pancreatic head. A lesion in the pancreatic head was reported to be an independent factor for the requirement for multiple needle passes[13].

There are two methods of puncturing the pancreatic head. Solid pancreatic head lesions are punctured in the duodenal bulbus by pushing (push method) or pulling (pull method) the echoendoscope after it is inserted into the second portion of the duodenum. It is not apparent which of the two methods is more effective. Therefore, we evaluated the efficacy of EUS-FNA of pancreatic head lesions in patients who underwent the push and/or pull method.

MATERIALS AND METHODS

Study design
In this retrospective study, we compared the efficacy of EUS-FNA using the push and pull methods for pancreatic head lesions. This study was approved by the Institutional Review Board of Fukushima Medical University.
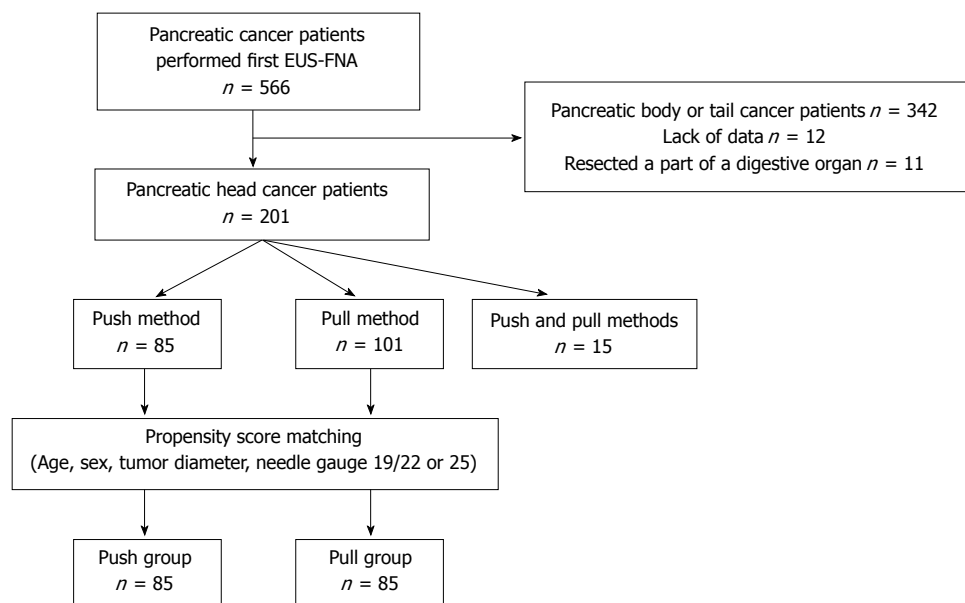
Figure 1 Subjects in this study. Propensity score matching was performed by using patient data [age, sex, tumor diameter, and needle gauge (19/22 or 25)]. After propensity score matching, 85 patients each were stratified into the two groups. EUS-FNA: Endoscopic ultrasound-guided fine needle aspiration.

Patients

In all, 566 pancreatic cancer patients underwent their first EUS-FNA between February 2001 and December 2017 at Fukushima Medical University (Figure 1). Among these patients, 201 who underwent EUS-FNA of the pancreatic head were included in this study. EUS-FNA was performed using the push method in 85 patients, the pull method in 101 patients, both the push and pull methods in 15 patients.

Because of the retrospective design of the study, the push or pull method was not randomly assigned. Therefore, propensity score matching was used to reduce selection bias derived from patient background (age, sex, tumor diameter, and FNA needle type—19/22 or 25 G). Regarding the FNA needle, a 19-G needle was used in only 3 patients. Therefore, propensity score matching was performed based on whether the needle was 25 G. The propensity score was calculated by using logistic regression analysis. After propensity score matching, 85 patients each were stratified into the push and pull groups.

EUS-FNA procedures

An echoendoscope was inserted into the patients after they were sufficiently sedated with midazolam. After the echoendoscope reached the antrum of the stomach or the duodenal bulbus, pancreatic head cancer was visualized by pushing the echoendoscope (push method). In contrast, when the echoendoscope reached the descending part of the duodenum, pancreatic head cancer was visualized by pulling the echoendoscope (pull method). After the lack of blood flow was confirmed on the puncture line by Doppler ultrasound, needle passes were started. Rapid onsite cytology (ROSE) of the EUS-FNA specimen was performed by the

cytoscreener[14-19]. If the amount of the specimen was sufficient, the procedure was completed. However, if the amount of specimen was insufficient, more punctures were performed until sufficient specimen was collected, as determined by the pathologist. Class IV and V disease was characterized as a malignancy by cytology. Histology specimens were fixed overnight in 10% formalin solution. Specimens were sectioned onto slides and stained with hematoxylin and eosin or prepared for immunostaining (p53 or Ki-67) as necessary. Patients not diagnosed with pancreatic cancer by EUS-FNA were eventually diagnosed by EUS-FNA of lymph node metastases, second EUS-FNA, surgery, pathological autopsy, or biliary juice cytology.

The echoendoscope used in this study was GF-Y0005-UCT, GF-UC240AL-5, GF-UCT240AL-5, or GF-UCT260 (Olympus Medical Systems, Tokyo, Japan). The ultrasonography equipment used in this study was EU-ME1 or EU-ME2 (Olympus Medical Systems) or SSD5000 or ALOKA ProSound α-10 (ALOKA, Tokyo, Japan). The biopsy needles were Expect 22 or 25 G or Acquire 22 G (Boston Scientific, MA, USA); EZ Shot 22 G, EZ Shot 2 22 G, EZ Shot 3 plus 22 G, or NA11J-KB (Olympus Medical System); EchoTip 19, 22, or 25 G or Quick-Core 19 G (Cook Medical Inc., NC, United States); or Sonotip 22 or 25 G (Medi-Globe GmbH, Achenmühle, Germany).

Examination items

Patient characteristics (age, sex, and tumor diameter) and factors related to EUS-FNA (needle gauge, number of needle passes, distance to lesion, diagnosis of malignancy by cytology, diagnosis of malignancy by histology, overall diagnosis of malignancy, and adverse events) were compared between the push and pull

Table 1 Comparison of patient characteristics and endoscopic ultrasound-guided fine needle aspiration results

	Push group (*n* = 85)	Pull group (*n* = 85)	*P* value
Patient characteristics			
Age (yr), mean ± SD	67.1 ± 10.2	67.6 ± 9.7	0.77
Sex (male/female)	48/37	46/39	0.88
Tumor diameter (mm), mean ± SD	25.9 ± 10.3	26.6 ± 10.1	0.67
EUS-FNA results			
Distance to lesion	13.9 ± 4.9	7.0 ± 4.9	< 0.01
No. of needle passes of pancreas, mean ± SD	2.4 ± 1.3	2.4 ± 1.3	0.86
Needle gauge			0.57
19	2	1	
22	32	38	
25	51	46	
Diagnosis of malignancy by cytology, *n* (%)	78 (91.8)	78 (91.8)	1
Diagnosis of malignancy by histology, *n* (%)	47 (55.3)	34 (40.0)	0.065
Overall diagnosis of malignancy, *n* (%)	80 (94.1)	80 (94.1)	1
Adverse events			
Abscess around pancreas, *n* (%)	0 (0)	1 (1.2)	1

EUS-FNA: Endoscopic ultrasound-guided fine needle aspiration.

Figure 2 Measurement of tumor diameter and distance to tumor. A: The tumor diameter was measured by endoscopic ultrasonography; B: The distance to the tumor was measured between the upper end of the puncture line and the tumor.

groups. Tumor diameter and the distance to lesion were measured by EUS (Figure 2).

Statistical analyses

Student's t test was used to compare continuous variables. Fisher's exact test was used to compare nominal variables. Logistic regression was used to investigate factors that influenced the histological diagnosis of malignancy using EUS-FNA specimens. A *P* value < 0.05 indicated a significant difference. All statistical analyses were performed using the EZR platform (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, EZR is a modified version of the R commander that was designed to perform functions frequently used in biostatistics[20].

RESULTS

Patient characteristics were not significantly different between the two groups (Table 1). Regarding the factors related to EUS-FNA, the distance to lesion was significantly longer in the push group than in the pull group (13.9 ± 4.9 mm *vs* 7.0 ± 4.9 mm, *P* < 0.01). Other factors were not different between the two groups.

We investigated factors influencing the distance to lesion [≥ 10 mm (median)]. The push method was identified as significant in the univariate analysis (Table 2). Because no factors with *P* < 0.15 were identified, a multivariate analysis was not performed.

We investigated factors influencing the histological diagnosis of malignancy. Among factors potentially influencing the histological diagnosis of malignancy, tumor diameter ≥ 25 mm and the push method were identified as significant in the univariate analysis (factors with *P* < 0.15 were selected for the multivariate analysis) (Table 3). Logistic regression was performed using these two items, and independent factors included tumor diameter ≥ 25 mm [odds ratio (OR) = 1.91, 95% confidence interval (CI): 1.02-3.58, *P* = 0.043] and the push method (OR = 1.91, 95%CI: 1.03-3.55, *P* = 0.04) (Table 4).

Table 2 Factors influencing the distance to lesion *n* (%)

	Distance to lesion < 10 mm (*n* = 70)	Distance to lesion ≥ 10 mm (*n* = 100)	*P* value
Age ≥ 68 yr	32 (45.7)	56 (56.0)	0.21
Sex, male	35 (50.0)	59 (59.0)	0.28
Tumor diameter ≥ 25 mm	27 (38.6)	45 (45.0)	0.43
Push method	12 (17.1)	73 (73.0)	< 0.01
Needle gauge, 19 or 22 G	32 (45.7)	41 (41.0)	0.64

Table 3 Factors contributing to the diagnosis of malignancy by histology (univariate analysis) *n* (%)

	Malignancy by histology(-) (*n* = 89)	Malignancy by histology(+) (*n* = 81)	*P* value
Age ≥ 68 yr	50 (56.2)	38 (46.9)	0.28
Sex, male	50 (56.2)	44 (54.3)	0.88
Tumor diameter ≥ 25 mm	45 (50.6)	53 (65.4)	0.062
Push method	38 (42.7)	47 (58.0)	0.065
Needle gauge, 19 or 22 G	37 (41.6)	36 (44.4)	0.76
Needle passes ≥ 2	71 (80.0)	59 (72.8)	0.37

Table 4 Factors contributing to the diagnosis of malignancy by histology (multivariate analysis)

	OR	95%CI	*P* value
Tumor diameter ≥ 25 mm	1.91	1.02-3.58	0.043
Push method	1.91	1.03-3.55	0.040

DISCUSSION

In this study, we investigated which method (push or pull method) of EUS-FNA was more effective in diagnosing pancreatic head cancer. The distance to lesion was significantly longer in the push group than in the pull group, and the push method was an independent factor contributing to the histological diagnosis of malignancy using EUS-FNA specimens.

As mentioned above, the ability to diagnose pancreatic masses by EUS-FNA has been reported to be excellent[3-8]. However, pancreatic head lesions were reported to be more difficult than lesions in other pancreatic locations to diagnose by EUS-FNA. Some reports have attempted to explain why EUS-FNA is difficult for pancreatic head lesions. One report indicated that pancreatic head lesions are visualized on the middle right of the EUS image, and the lesion is easily moved[21]. Another report suggested that because the tip of the echoendoscope is flexed, the passage of the needle is more difficult[22]. In this report, pancreatic head lesions were closer to the starting point when the needle was inserted by the pull method; however, the overall diagnosis of malignancy was not different between the push and pull groups. Thus, if a diagnosis of malignancy is needed or if the endoscopist is inexperienced, EUS-FNA of pancreatic head lesions should be performed by using the pull method.

The pull method better facilitates EUS-FNA for pancreatic head lesions than the push method; however, it has been reported that the echoendoscope position can

become unstable[22]. In this report, the push method was an independent factor contributing to the histological diagnosis of pancreatic cancer. The direction in which the echoendoscope is pulled is opposite that of the lesion puncture, whereas the direction in which the echoendoscope is pushed is the same as that of lesion puncture. In addition, the echoendoscope is stable in the push method. These factors might explain why the push method contributed to the diagnosis of malignancy by histology of the EUS-FNA specimen. Therefore, the push method is recommended for patients requiring histology of EUS-FNA specimens, for example, patients who have a medical history of other cancers.

This study has some limitations. First, this study was retrospective and performed in a single center. We used propensity scoring to overcome this limitation; however, prospective and large-scale studies are warranted. Second, due to the retrospective nature of this study, EUS-FNA procedures were not performed by specific endoscopists. In this study, EUS-FNA was performed by specialists who had performed > 3000 pancreaticobiliary EUS procedures or by trainees under the guidance of specialists. Therefore, the quality of the EUS procedure was considered constant. Third, the difficulty of each method was not evaluated by Doppler ultrasound of blood flow on the puncture line. Because of the retrospective nature of this study, we were unable to identify the potential difficulty of either method by Doppler ultrasound of blood flow. Fourth, we used an EUS-guided trucut biopsy (EUS-TCB) needle or an EUS-guided fine needle biopsy (FNB) needle, such as the Quick-Core 19 G (Cook Medical Inc.) or Acquire 22 G needle (Boston Scientific). Although these needles have been reported to increase the yield of samples[23,24], EUS-TCB and EUS-FNB needles were used in only two patients. A Quick-Core needle (Cook Medical Inc.) was used in a patient in the push group, and an Acquire needle (Boston Scientific) was used in a patient in the pull group. Therefore, we do not believe that the use of

EUS-TCB and EUS-FNB needles significantly influenced the results.

In conclusion, the pull method shortened the distance between the endoscope and the lesion and facilitated EUS-FNA of pancreatic head cancer. The push method contributed to the histological diagnosis of pancreatic head cancer using EUS-FNA specimens.

ARTICLE HIGHLIGHTS

Research background
Endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) plays an important role in the diagnosis of pancreatic cancer. However, EUS-FNA of pancreatic head lesions is difficult. The pull and push methods are both used to diagnose pancreatic head lesions. It is unknown which method is more efficient.

Research motivation
We wanted to determine the appropriate puncture method for EUS-FNA of pancreatic head lesions.

Research objectives
The primary objective of this study was to reveal which method (push method or pull method) is more efficient for diagnosing pancreatic head cancer.

Research methods
We placed 85 patients in each group (push group and pull group) using propensity score matching. Patient characteristics and some EUS-FNA-related factors were compared between the push and pull groups.

Research results
The distance to the pancreatic cancer was significantly longer in the push group than in the pull group. The push method was identified as a significant factor contributing to the histological diagnosis of malignancy.

Research conclusions
The pull method shortened the distance between the echoendoscope and the lesion and facilitated EUS-FNA of pancreatic head cancer. The push method contributed to the histological diagnosis of pancreatic head cancer using EUS-FNA specimens.

Research perspectives
If only a diagnosis of malignancy is needed or if the endoscopist is inexperienced, the pull method is recommended. However, the push method is recommended for patients requiring histological analysis of EUS-FNA specimens. Further prospective and large-scale studies on these methods are warranted.

REFERENCES

1   **DeWitt J**, Devereaux B, Chriswell M, McGreevy K, Howard T, Imperiale TF, Ciaccia D, Lane KA, Maglinte D, Kopecky K, LeBlanc J, McHenry L, Madura J, Aisen A, Cramer H, Cummings O, Sherman S. Comparison of endoscopic ultrasonography and multidetector computed tomography for detecting and staging pancreatic cancer. *Ann Intern Med* 2004; **141**: 753-763 [PMID: 15545675 DOI: 10.7326/0003-4819-141-10-200411160-00006]

2   **Tamm EP**, Loyer EM, Faria SC, Evans DB, Wolff RA, Charnsangavej C. Retrospective analysis of dual-phase MDCT and follow-up EUS/EUS-FNA in the diagnosis of pancreatic cancer. *Abdom Imaging* 2007; **32**: 660-667 [PMID: 17712589 DOI: 10.1007/s00261-007-9298-x]

3   **Williams DB**, Sahai AV, Aabakken L, Penman ID, van Velse A, Webb J, Wilson M, Hoffman BJ, Hawes RH. Endoscopic ultrasound guided fine needle aspiration biopsy: a large single centre experience. *Gut* 1999; **44**: 720-726 [PMID: 10205212]

4   **Eloubeidi MA**, Jhala D, Chhieng DC, Chen VK, Eltoum I, Vickers S, Mel Wilcox C, Jhala N. Yield of endoscopic ultrasound-guided fine-needle aspiration biopsy in patients with suspected pancreatic carcinoma. *Cancer* 2003; **99**: 285-292 [PMID: 14579295 DOI: 10.1002/cncr.11643]

5   **Ryozawa S**, Kitoh H, Gondo T, Urayama N, Yamashita H, Ozawa H, Yanai H, Okita K. Usefulness of endoscopic ultrasound-guided fine-needle aspiration biopsy for the diagnosis of pancreatic cancer. *J Gastroenterol* 2005; **40**: 907-911 [PMID: 16211348 DOI: 10.1007/s00535-005-1652-6]

6   **Yoshinaga S**, Suzuki H, Oda I, Saito Y. Role of endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) for diagnosis of solid pancreatic masses. *Dig Endosc* 2011; **23** Suppl 1: 29-33 [PMID: 21535197 DOI: 10.1111/j.1443-1661.2011.01112.x]

7   **Uehara H**, Ikezawa K, Kawada N, Fukutake N, Katayama K, Takakura R, Takano Y, Ishikawa O, Takenaka A. Diagnostic accuracy of endoscopic ultrasound-guided fine needle aspiration for suspected pancreatic malignancy in relation to the size of lesions. *J Gastroenterol Hepatol* 2011; **26**: 1256-1261 [PMID: 21501226 DOI: 10.1111/j.1440-1746.2011.06747.x]

8   **Mohammad Alizadeh AH**, Shahrokh S, Hadizadeh M, Padashi M, Zali MR. Diagnostic potency of EUS-guided FNA for the evaluation of pancreatic mass lesions. *Endosc Ultrasound* 2016; **5**: 30-34 [PMID: 26879164 DOI: 10.4103/2303-9027.175879]

9   **Singh S**, Purohit T, Aoun E, Patel Y, Carleton N, Mitre M, Morrissey S, Dhawan M, Thakkar S. Comparison of the outcomes of endoscopic ultrasound based on community hospital versus tertiary academic center settings. *Dig Dis Sci* 2014; **59**: 1925-1930 [PMID: 24573718 DOI: 10.1007/s10620-014-3075-9]

10 **Haba S**, Yamao K, Bhatia V, Mizuno N, Hara K, Hijioka S, Imaoka H, Niwa Y, Tajika M, Kondo S, Tanaka T, Shimizu Y, Yatabe Y, Hosoda W, Kawakami H, Sakamoto N. Diagnostic ability and factors affecting accuracy of endoscopic ultrasound-guided fine needle aspiration for pancreatic solid lesions: Japanese large single center experience. *J Gastroenterol* 2013; **48**: 973-981 [PMID: 23090002 DOI: 10.1007/s00535-012-0695-8]

11 **Tadic M**, Kujundzic M, Stoos-Veic T, Kaic G, Vukelic-Markovic M. Role of repeated endoscopic ultrasound-guided fine needle aspiration in small solid pancreatic masses with previous indeterminate and negative cytological findings. *Dig Dis* 2008; **26**: 377-382 [PMID: 19188731 DOI: 10.1159/000177025]

12 **Varadarajulu S**, Tamhane A, Eloubeidi MA. Yield of EUS-guided FNA of pancreatic masses in the presence or the absence of chronic pancreatitis. *Gastrointest Endosc* 2005; **62**: 728-736; quiz 751, 753 [PMID: 16246688 DOI: 10.1016/j.gie.2005.06.051]

13 **Uehara H**, Sueyoshi H, Takada R, Fukutake N, Katayama K, Ashida R, Ioka T, Takenaka A, Nagata S, Tomita Y. Optimal number of needle passes in endoscopic ultrasound-guided fine needle aspiration for pancreatic lesions. *Pancreatology* 2015; **15**: 392-396 [PMID: 25979252 DOI: 10.1016/j.pan.2015.04.005]

14 **Logroño R**, Waxman I. Interactive role of the cytopathologist in EUS-guided fine needle aspiration: an efficient approach. *Gastrointest Endosc* 2001; **54**: 485-490 [PMID: 11577312]

15 **Klapman JB**, Logrono R, Dye CE, Waxman I. Clinical impact of on-site cytopathology interpretation on endoscopic ultrasound-guided fine needle aspiration. *Am J Gastroenterol* 2003; **98**: 1289-1294 [PMID: 12818271 DOI: 10.1111/j.1572-0241.2003.07472.x]

16   **Erickson RA**. EUS-guided FNA. *Gastrointest Endosc* 2004; **60**: 267-279 [PMID: 15278063 DOI: 10.1016/S0016-5107(04)01529-9]

17   **Yamao K**, Sawaki A, Mizuno N, Shimizu Y, Yatabe Y, Koshikawa T. Endoscopic ultrasound-guided fine-needle aspiration biopsy (EUS-FNAB): past, present, and future. *J Gastroenterol* 2005; **40**: 1013-1023 [PMID: 16322944 DOI: 10.1007/s00535-005-1717-6]

18   **Hikichi T**, Irisawa A, Bhutani MS, Takagi T, Shibukawa G, Yamamoto G, Wakatsuki T, Imamura H, Takahashi Y, Sato A, Sato M, Ikeda T, Hashimoto Y, Tasaki K, Watanabe K, Ohira H, Obara K. Endoscopic ultrasound-guided fine-needle aspiration of solid pancreatic masses with rapid on-site cytological evaluation by endosonographers without attendance of cytopathologists. *J Gastroenterol* 2009; **44**: 322-328 [PMID: 19274426 DOI: 10.1007/s00535-009-0001-6]

19   **Dumonceau JM**, Koessler T, van Hooft JE, Fockens P. Endoscopic ultrasonography-guided fine needle aspiration: Relatively low sensitivity in the endosonographer population. *World J Gastroenterol* 2012; **18**: 2357-2363 [PMID: 22654426 DOI: 10.3748/wjg.v18.i19.2357]

20   **Kanda Y**. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant* 2013; **48**: 452-458 [PMID: 23208313 DOI: 10.1038/bmt.2012.244]

21   **Yasuda I**, Iwashita T, Doi S. Tips for endoscopic ultrasound-guided fine needle aspiration of various pancreatic lesions. *J Hepatobiliary Pancreat Sci* 2014; **21**: E29-E33 [PMID: 24353093 DOI: 10.1002/jhbp.60]

22   **Kedia P**, Gaidhane M, Kahaleh M. Technical Advances in Endoscopic Ultrasound (EUS)-Guided Tissue Acquisition for Pancreatic Cancers: How Can We Get the Best Results with EUS-Guided Fine Needle Aspiration? *Clin Endosc* 2013; **46**: 552-562 [PMID: 24143320 DOI: 10.5946/ce.2013.46.5.552]

23   **Thomas T**, Kaye PV, Ragunath K, Aithal G. Efficacy, safety, and predictive factors for a positive yield of EUS-guided Trucut biopsy: a large tertiary referral center experience. *Am J Gastroenterol* 2009; **104**: 584-591 [PMID: 19262518 DOI: 10.1038/ajg.2008.97]

24   **Mukai S**, Itoi T, Yamaguchi H, Sofuni A, Tsuchiya T, Tanaka R, Tonozuka R, Honjo M, Fujita M, Yamamoto K, Matsunami Y, Asai Y, Kurosawa T, Nagakawa Y. A retrospective histological comparison of EUS-guided fine-needle biopsy using a novel franseen needle and a conventional end-cut type needle. *Endosc Ultrasound* 2018; Epub ahead of print [PMID: 29786033 DOI: 10.4103/eus.eus_11_18]

Liver intravoxel incoherent motion diffusion-weighted imaging for the assessment of hepatic steatosis and fibrosis in children

Hyun Joo Shin, Haesung Yoon, Myung-Joon Kim, Seok Joo Han, Hong Koh, Seung Kim, Mi-Jung Lee

Hyun Joo Shin, Haesung Yoon, Myung-Joon Kim, Mi-Jung Lee, Department of Radiology, Severance Hospital, Severance Pediatric Liver Disease Research Group, Research Institute of Radiological Science, Yonsei University College of Medicine, Seoul 03722, South Korea

Seok Joo Han, Department of Surgery, Severance Hospital, Severance Pediatric Liver Disease Research Group, Yonsei University College of Medicine, Seoul 03722, South Korea

Hong Koh, Seung Kim, Division of Gastroenterology, Hepatology and Nutrition, Department of Pediatrics, Severance Children's Hospital, Severance Pediatric Liver Disease Research Group, Yonsei University College of Medicine, Seoul 03722, South Korea

ORCID number: Hyun Joo Shin (0000-0002-7462-2609); Haesung Yoon (0000-0003-0581-8656); Myung-Joon Kim (0000-0002-4608-0275); Seok Joo Han (0000-0001-5224-1437); Hong Koh (0000-0002-3660-7483); Seung Kim (0000-0003 -4373-9828); Mi-Jung Lee (0000-0003-3244-9171).

Author contributions: Shin HJ and Lee MJ designed the research; Shin J, Yoon H, and Lee MJ performed the research and wrote the manuscript; Shin HJ, Yoon H and Lee MJ analyzed the data; Kim MJ, Han SJ, Koh H, and Kim S contributed analytic tools; Kim MJ, Shin HJ, Yoon H, Han SJ, Koh H, Kim S, and Lee MJ revised and approved the final version.

Institutional review board statement: This study was reviewed and approved by the local ethics committee of the Severance Hospital, Yonsei University (register no. 1-2017-0014).

Informed consent statement: Because of the retrospective and anonymous character of this study, the need for informed consent was waived by the institutional review board.

Conflict-of-interest statement: All authors declare no conflicts-of-interest related to this article.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was

Abstract

AIM

To evaluate the correlation between intravoxel incoherent motion (IVIM) diffusion-weighted imaging (DWI) parameters and the degree of hepatic steatosis and fibrosis in children.

METHODS

This retrospective study was approved by the institutional review board. The children (≤ 18 years) who underwent liver IVIM DWI with 8 *b*-values under

the suspicion of hepatic steatosis or fibrosis from February 2013 to November 2016 were included. Subjects were divided into normal, fatty liver (FAT), and fibrotic liver (FIB) groups. The slow diffusion coefficient (D), fast diffusion coefficient (D*), perfusion fraction (f), and apparent diffusion coefficient (ADC) were measured. MR proton density fat fraction (PDFF), MR elastography (MRE), and IVIM values were compared.

RESULTS
A total of 123 children (median age of 12 years old, range: 6-18 years) were included, with 8 in the normal group, 93 in the FAT group, and 22 in the FIB group. The D* values were lower in the FIB group compared with those of the normal ($P = 0.015$) and FAT ($P = 0.003$) groups. The f values were lower in the FIB group compared with the FAT group ($P = 0.001$). In multivariate analyses, PDFF value was positively correlated with f value ($\beta = 3.194$, $P < 0.001$), and MRE value was negatively correlated with D* value ($\beta = -7.031$, $P = 0.032$). The D and ADC values were not influenced by PDFF or MRE value.

CONCLUSION
In liver IVIM DWI with multiple *b*-values in children, there was a positive correlation between hepatic fat and blood volume, and a negative correlation between hepatic stiffness and endovascular blood flow velocity, while diffusion-related parameters were not affected.

Key words: Intravoxel incoherent motion; Diffusion-weighted imaging; Fibrosis; Fatty liver; Pediatrics

Core tip: Liver intravoxel incoherent motion (IVIM) diffusion-weighted imaging with multiple *b*-values was feasible and useful for evaluating hepatic steatosis and fibrosis in children. Hepatic steatosis and fibrosis affected IVIM parameters differently in children. MR proton density fat fraction (PDFF) demonstrated a positive correlation with the perfusion fraction (f) of IVIM. MR elastography (MRE) value was negatively correlated with the fast diffusion coefficient (D*) of IVIM. The other IVIM values were not influenced by PDFF or MRE value.

INTRODUCTION

Liver disease in the pediatric population is not uncommon. Metabolic disease and diverse syndromic conditions commonly affect the liver to varying degrees. Nonalcoholic fatty liver disease (NAFLD) is an important disease entity because it could lead to hepatic steatosis and fibrosis[1]. In addition, the incidence of NAFLD is increasing dramatically in adolescents and in children[1]. Biliary atresia (BA) or neonatal hepatitis also can affect the liver in early childhood, and BA is the most common cause of hepatic fibrosis and reason for liver transplantation in young children[2,3]. Therefore, quantitative and non-invasive characterization of the liver conditions is important in pediatric patients. Biopsy has a limited role in children due to its sampling bias and invasive nature[4]. The intravoxel incoherent motion (IVIM) magnetic resonance imaging (MRI) technique could be one of the alternative methods for assessing hepatic tissue perfusion and diffusion separately in various pediatric liver diseases.

The IVIM technique separates the slow and fast diffusion components of diffusion-weighted imaging (DWI)[5]. The slow component is related to free water diffusion, and the fast component is related to microvascular perfusion[5]. Although the conventional apparent diffusion coefficient (ADC) is influenced by both slow and fast components, IVIM could quantify the two components separately by using multiple *b*-values[6]. It is because perfusion property has fast diffusion rate while using low *b*-values, but pure water molecular diffusion has slow diffusion rate while using high number of *b*-values[6]. Three parameters can be obtained from the IVIM technique: (1) Pure molecular diffusion (D, D slow), which is related to the slow component of diffusion from water; (2) pseudo-diffusion (D*, D fast), which reflects the endovascular blood flow velocity; and (3) perfusion fraction (f), which reflects blood volume[5]. Among them, D* and f are related to blood perfusion.

Many studies have utilized IVIM for the evaluation of liver disease in adults. They demonstrated the better performance of IVIM compared to DWI for staging hepatic steatosis and fibrosis, and fibrosis had different effects on the IVIM parameters[7-9]. However, limited studies included children for assessing liver disease using IVIM. One study included patients 10 years of age or older to evaluate diminished liver microperfusion after Fontan operation[10]. Another study included patients 11 years of age or older to assess the correlations between hepatic steatosis and IVIM perfusion parameters[11]. In the most recent study, Manning *et al*[12] evaluated D, f, and ADC values derived from three *b*-values for assessing NAFLD in children. However, there has been no study focused on pediatric livers with enough *b*-values for IVIM.

Therefore, the purpose of this study was to evaluate the correlation between IVIM DWI parameters and the degree of hepatic steatosis and fibrosis measured with MRI in children.

MATERIALS AND METHODS

Subjects
Our institutional review board approved this retro-

Table 1 Liver magnetic resonance imaging parameters				
	SSFSE T2 weighted image	IDEAL-IQ for PDFF and R2* (1/T2*) map	MRE	IVIM
Acquisition plane	Axial	Axial	Axial	Axial
Acquisition scheme	Breath hold	Breath hold	Breath hold	Free breathing
TR (millisecond)	540	5.9	1000	6000
TE (millisecond)	80	2.6	62	74
Acquisition matrix	320 × 256	128 × 128	64 × 64	128 × 128
Field of view (cm)	40	42	38	38
Slice/internal thickness (mm)	8	8	8	4
Gap (mm)	10	8	2	5
Flip angle (°)	90	3	90	90
Number of signal averages	0.64	0.69	2	2 → 1
Notes		Six gradient echoes (from 0.9 to 4.4 millisecond)	60 Hz MEG (20% driver power reduction)	Eight *b*-values (15, 27.3, 49.8, 90.7, 165.3, 301.2, 548.8, 1000)
Scan time (min)	0:12	0:16	0:24 (or 0:12 × 2 times)	4:30

SSFSE: Single shot fast spine echo; PDFF: MR proton density fat fraction; MRE: MR elastography; IVIM: Intravoxel incoherent motion; TR: Repetition time; TE: Echo time; MEG: Motion encoding gradient.

spective study, and informed consent was waived. Children under the age of 18 years who underwent liver MRI with IVIM DWI from February 2013 to November 2016 were included. There were only two clinical indications: Patients with clinical suspicion of hepatic steatosis or those who were suspected to have hepatic fibrosis after undergoing Kasai operation due to BA. The exclusion criteria were: (1) Subjects who had no laboratory results of aspartate aminotransferase (AST) or alanine aminotransferase (ALT) level within one month of the MRI examination and (2) those who had focal lesions other than fat or fibrosis in the liver, such as hemangioma or cysts on MRI.

Subjects were divided into three groups. One was the normal group, for those who had normal MR proton density fat fraction (PDFF) of the liver (< 6% according to a previous study)[13], normal body mass index, and normal transient elastography (TE) results when they had TE results within one year of MRI examinations. The second was the fatty liver (FAT) group, for subjects who had PDFF greater than 6%. The third was the fibrotic liver (FIB) group, for children who underwent MRI after a Kasai operation due to BA. The age, gender, weight (kg), and height (cm) of the children were assessed at the time of MRI examination.

MRI analysis
Each liver MRI was obtained in a 3T system (Discovery 750w, GE Healthcare, Milwaukee, WI, United States). MRI was composed of four sequences including axial single shot fast spine echo (SSFSE) T2 weighted images, 3D volumetric multi-echo gradient sequence (IDEAL-IQ, for proton density fat quantification and T2* decay assessment), MR elastography (MRE), and IVIM DWI. The detailed MR parameters are presented in Table 1. For MRE, a passive pneumatic driver was placed over the liver and was connected to the active driver[14]. The driver power was reduced 20% from the adult level, and a folded towel was placed between the driver

and abdominal wall to avoid abdominal discomfort[15,16].

In the SSFSE T2 weighted images, liver morphology and focal lesions were assessed. Round regions-of-interest (ROIs) were drawn in the homogenous parenchyma of the right hepatic lobe, avoiding hepatic vessels, by one experienced radiologist in all quantitative imaging sequences. Four ROIs were drawn in the liver parenchyma in each slice, and the mean values were considered as representative values in each sequence, including D ($\times 10^{-3}$ mm^2/s), D* ($\times 10^{-3}$ mm^2/s), *f* (%), and ADC ($\times 10^{-3}$ mm^2/s) value maps (Figure 1).

Statistical analysis
Statistical analyses were performed using SPSS version 23 (IBM Corp., Armonk, NY, United States). Clinical findings and MRI values were compared between normal, FAT and FIB groups using Kruskal-Wallis test or Fisher's exact test, with the use of Dunn's method for multiple comparisons. We also performed linear regression analysis to determine whether the liver function tests, PDFF, and MRE values influenced the IVIM parameters. All data were presented as median values and interquartile ranges. *P*-values less than 0.05 were considered statistically significant for all analyses.

RESULTS
During the study period, a total of 130 children (M: F = 86:44) with a median age of 12.2 years (range: 6-18 years) underwent liver MRI including IVIM DWI. From them, seven children were excluded because four had no laboratory results within one month of the MRI, and three had focal hepatic lesions such as hepatic hemangioma or cysts. Therefore, 123 children were ultimately included in this study. There were 83 boys and 40 girls, and the median age was 12 years, with a range of 6-18 years. Among these, 101 children underwent MRI under the suspicion of
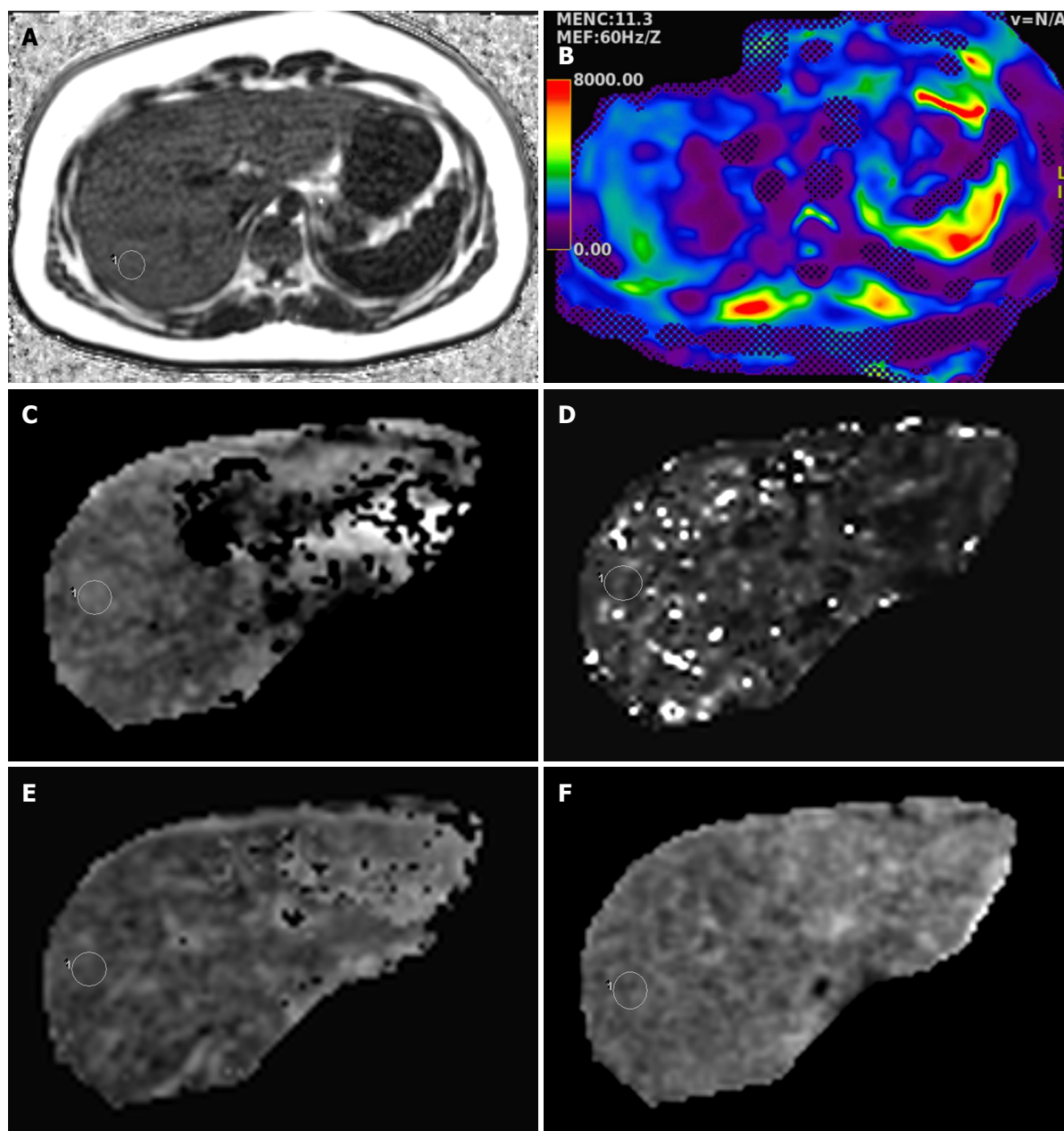
Figure 1 Magnetic resonance imaging of liver from a 16-year-old boy in the fatty liver group. A: MRI-proton density fat fraction = 17.6%; B: MR elasticity = 3.2 kPa; C: D value = 1.45×10^{-3} mm^2/s; D: D* value = 90×10^{-3} mm^2/s; E: *f* value = 31.8%; F: ADC value = 1.32×10^{-3} mm^2/s.

hepatic steatosis. According to the grouping criteria, eight children were included in the normal group and 93 children were included in the FAT group. During the study period, 22 children underwent MRI after Kasai operation due to BA and were included in the FIB group.

Comparison of clinical findings and MRI parameters between the three groups

The group comparison results are summarized in Table 2. Most clinical parameters did not differ between the normal and FAT groups or between the normal and FIB groups. However, the FIB group had younger children and more girls than the FAT group. The weight was higher in the FAT group than in the normal group. The

AST and ALT values were significantly higher in the FAT group compared to the normal group.

The median PDFF values were higher in the FAT group (22.0 %) than in the normal (2.7 %) and FIB (2.6 %) groups ($P < 0.001$ for both). The median T2* values were lower in the FAT group (18.0 msec) compared with the normal group (25.8 msec, $P = 0.007$) and the FIB group (23.4 msec, $P < 0.001$). In addition, the median MRE values were higher in the FIB group (3.2 kPa) compared with the normal group (2 kPa, $P < 0.001$) and the FAT group (2.6 kPa, $P = 0.001$).

From the IVIM parameters, the D and ADC values were not significantly different in the three groups. In contrast, the median D* values were significantly lower in the FIB group (36.5×10^{-3} mm^2/s) compared

Table 2 Comparison of clinical and magnetic resonance imaging findings between the three groups

		Normal (*n* = 8)[1]	FAT (*n* = 93)[1]	FIB (*n* = 22)[1]	*P*-value[2]	*P*-value[3]	*P*-value[4]	*P*-value[5]
Clinical findings	Age (yr)	11 (9.25-13.75)	12 (10-15.5)	10 (8.75-11)	< 0.001[a]	0.611	0.855	< 0.001[a]
	Gender (M:F)	4:04	70:23:00	9:13	0.005[a]	0.615	1.000	0.012[a]
	Weight (kg)	36.5 (28.5-49.5)	65 (51.9-76.5)	33 (28.3-44)	< 0.001[a]	0.001[a]	1.000	< 0.001[a]
	Height (cm)	145 (137-162)	158 (145-164)	136.3 (131.8-148.1)	< 0.001[a]	0.543	0.563	< 0.001[a]
	AST (IU/L)	22.5 (18.3-24.8)	52.0 (27.0-80.5)	33.0 (22.8-68.0)	0.007[a]	0.008[a]	0.167	0.541
	ALT (IU/L)	11.0 (9.0-12.0)	87.0 (44.5-138.5)	28.0 (16.0-55.3)	< 0.001[a]	< 0.001[a]	0.533	< 0.001[a]
MRI findings	PDFF (%)	2.65 (2.4-3.1)	22 (14.1-34)	2.6 (2.0-4.1)	< 0.001[a]	< 0.001[a]	1.000	< 0.001[a]
	T2* (millisecond)	25.8 (22.0-27.3)	18 (16.1-20.75)	23.4 (21-26.6)	< 0.001[a]	0.007[a]	1.000	< 0.001[a]
	MRE (kPa)[6]	2.0 (1.6-2.5)	2.6 (2.2-3.1)	3.2 (2.8-4.6)	< 0.001[a]	0.058	< 0.001[a]	0.001[a]
	D ($\times 10^{-3}$ mm²/s)	1.06 (1.03, 1.1)	1.07 (0.94, 1.35)	0.93 (0.81, 1.2)	0.158			
	D* ($\times 10^{-3}$ mm²/s)	70.2 (44.3, 139.7)	61 (39.5, 81.2)	36.5 (19.2, 52.5)	0.001[a]	0.937	0.015[a]	0.003[a]
	f (%)	28.5 (18.6, 34.8)	31.5 (27.9, 39.3)	19.3 (14.0, 33.2)	0.001[a]	0.542	1.000	< 0.001[a]
	ADC ($\times 10^{-3}$ mm²/s)	1.08 (0.97, 1.2)	1.05 (0.9, 1.21)	0.97 (0.89, 1.15)	0.575			

[a]*P* < 0.05; [1]All values: Median (Q1-Q3); [2]Overall *P*-value from Kruskal-Wallis test or Fisher's exact test, after testing with Dunn's method for multiple comparisons; [3]Normal *vs* FAT groups; [4]Normal *vs* FIB groups; [5]FAT *vs* FIB groups; [6]*n* = 7, 77, 19 for the normal, FAT, and FIB groups, respectively. FAT: Fatty liver; FIB: Fibrotic liver; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PDFF: MR proton density fat fraction; MRE: MR elastography; ADC: Apparent diffusion coefficient.

Table 3 Univariate linear regression analysis for intravoxel incoherent motion parameters in all groups

	D ($\times 10^{-3}$ mm²/s)			D* ($\times 10^{-3}$ mm²/s)			*f* (%)			ADC ($\times 10^{-3}$ mm²/s)		
	β	SE	*P* value	β	SE	*P* value	β	SE	*P* value	β	SE	*P* value
AST (IU/L)	-1.389	0.588	0.020[a]	-0.145	0.072	0.047[a]	-0.238	0.266	0.372	-1.146	0.551	0.040[a]
ALT (IU/L)	-0.304	0.316	0.339	-0.030	0.039	0.445	0.096	0.141	0.496	-0.446	0.294	0.132
PDFF (%)	1.785	1.949	0.362	0.255	0.237	0.284	4.289	0.775	< 0.001[a]	-0.263	1.826	0.886
T2* (millisecond)	-8.400	4.042	0.040[a]	0.818	0.495	0.101	-5.002	1.767	0.005[a]	-4.978	3.814	0.194
MRE (kPa) (*n* = 103)	-58.819	22.493	0.010[a]	-8.582	2.672	0.002[a]	-27.982	7.408	< 0.001[a]	-33.675	20.407	0.102

[a]*P* < 0.05; IVIM: Intravoxel incoherent motion; ADC: Apparent diffusion coefficient; β: Coefficient; SE: Standard error of the estimated coefficient; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PDFF: MR proton density fat fraction; MRE: MR elastography.

with those of the normal group (70.2 $\times 10^{-3}$ mm²/s, *P* = 0.015) and the FAT group (61.0 $\times 10^{-3}$ mm²/s, *P* = 0.003). The median *f* values were significantly lower only in the FIB group compared with the FAT group (19.3% *vs* 31.5%, *P* = 0.001).

Assessment of effects of laboratory and MRI results on IVIM parameters in all groups
In univariate linear regression analysis, the D values demonstrated negative correlation with AST (*P* = 0.020), T2* (*P* = 0.040), and MRE (*P* = 0.010) values (Table 3). The D* values were also negatively correlated with AST (*P* = 0.047) and MRE (*P* = 0.002) values. The *f* values were affected positively by PDFF values (*P* < 0.001) and negatively by T2* and MRE values (*P* = 0.005 and *P* < 0.001, respectively). In addition, the AST results were associated with decreased ADC values (*P* = 0.004), while other MR parameters did not influence ADC values.

In multivariate linear regression analysis, neither AST nor ALT affected IVIM parameters (Table 4). Increased PDFF value was a significant independent factor for increasing *f* value (β = 3.194, *P* < 0.001, Figure 2), which meant a positive correlation between hepatic fat and blood volume. The T2* values affected D values negatively (*P* = 0.019) and D* values positively (*P* = 0.022). In addition, increased MRE values were a significant independent factor for decreasing D* values

(β = -7.031, *P* = 0.032, Figure 3), which indicated a negative correlation between hepatic elasticity and endovascular blood flow velocity. However, the values of the diffusion-related parameters D and ADC were not significantly influenced by PDFF or MRE values.
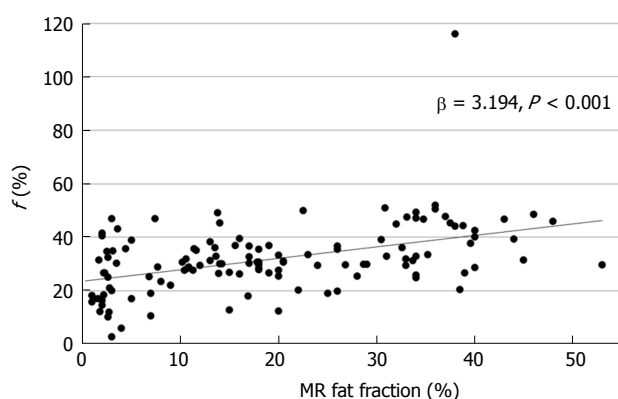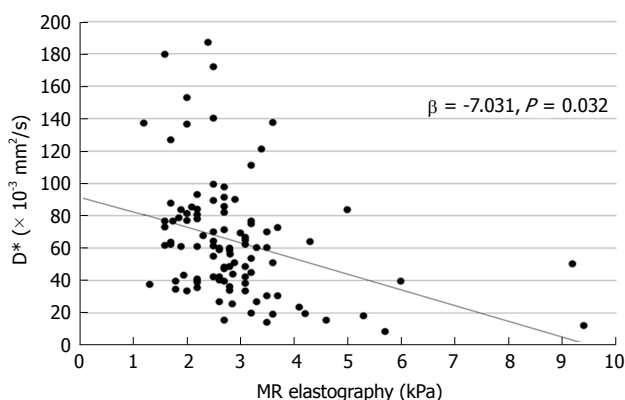
DISCUSSION

The IVIM technique has been used for kidney and liver diseases in a few previous studies of children[10-12,17,18]. Among the studies that included children, only one included only a pediatric population for assessing the usefulness of liver IVIM technique using three *b*-values in pathologically proven NAFLD[12]. In addition, there was no study that evaluated the meanings of IVIM parameters only in children, including normal, fatty, and fibrotic livers with sufficient *b*-values for IVIM, as in our study.

Our study is meaningful because we demonstrated the feasibility of IVIM with multiple *b*-values in pediatric liver MRI. The ages of the included children ranged from 6 to 18 years, younger than those in previous studies, and the included number of children was large. In addition, multiple *b*-values are needed to differentiate slow and fast diffusion with higher robustness and accuracy[19,20], because fast diffusion (D*) can be differentiated from low *b*-values, and slow diffusion (D)

Table 4 Multivariate linear regression analysis for intravoxel incoherent motion parameters in all groups

	D (× 10⁻³ mm²/s)			D* (× 10⁻³ mm²/s)			f (%)			ADC (× 10⁻³ mm²/s)		
	β	SE	*P* value	β	SE	*P* value	β	SE	*P* value	β	SE	*P* value
AST (IU/L)	-2.222	1.337	0.100	-0.063	0.161	0.697	-0.485	0.408	0.238	-1.343	1.227	0.277
ALT (IU/L)	0.718	0.673	0.289	0.013	0.081	0.877	0.116	0.206	0.574	0.419	0.618	0.499
PDFF (%)	-1.228	2.622	0.640	0.381	0.315	0.230	3.194	0.801	< 0.001ᵃ	-3.663	2.407	0.131
T2* (millisecond)	-11.189	4.697	0.019ᵃ	1.316	0.565	0.022ᵃ	-0.847	1.435	0.557	-8.473	4.313	0.052
MRE (kPa) (*n* = 103)	-37.042	26.853	0.171	-7.031	3.231	0.032ᵃ	-11.667	8.206	0.158	-29.679	24.658	0.232

ᵃ$P < 0.05$; IVIM: Intravoxel incoherent motion; ADC: Apparent diffusion coefficient; β: Coefficient; SE: Standard error of the estimated coefficient; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PDFF: MR proton density fat fraction; MRE: MR elastography.

Figure 2 Scatter plot of MR fat fraction and *f* value, showing a positive correlation.

Figure 3 Scatter plot of MR elasticity and D* value, showing a negative correlation.

can be differentiated from high *b*-values. Therefore, our methods using multiple *b*-values could lead to more accurate quantification and separation of IVIM parameters than previous studies.

The results revealed that hepatic steatosis and fibrosis influenced perfusion and diffusion parameters differently, while ADC did not show any significant correlation for these conditions; these results were concordant with previous studies[7,8]. In our study, increased PDFF value was a significant independent factor for increasing *f* value, which meant a positive correlation between hepatic steatosis and blood volume in pediatric NAFLD. In addition, increased MRE value was

a significant independent factor for decreasing D* value, which signified a negative correlation between hepatic fibrosis and endovascular blood flow velocity in children with BA. We performed multivariate logistic regression tests including liver function tests and PDFF and MRE values because liver inflammation, steatosis, and fibrosis could together influence histopathologic changes of the liver and slow and fast diffusions, and we could not obtain pathologic results from the included children.

In the previous studies, the patterns of increasing or decreasing IVIM parameters with hepatic steatosis or fibrosis varied. A previous study by Manning *et al*[12] revealed that hepatic steatosis and fibrosis had different effects on IVIM parameters, even though the relationships between parameters and hepatic conditions were different from those in our study. They showed decreased ADC and D values with increasing hepatic steatosis, while they observed decreased *f* values with increasing hepatic fibrosis in pediatric NAFLD. In hepatic steatosis, there are also studies that demonstrated a positive correlation between hepatic steatosis and *f* value in adults with NAFLD[21] and diffuse liver disease[6], similar to our results. Yu *et al*[22] also demonstrated the relationships between histopathologic change and IVIM parameters resulting in a positive correlation between hepatic steatosis and not only f, but also D value in a rat model of NAFLD. On the other hand, there is a study that demonstrated no correlation between IVIM parameters and the degree of hepatic steatosis in adults with various chronic liver diseases[23]. Franca *et al*[6] mentioned that *f* value was increased more prominently in a mild hepatic steatosis group than in a severe steatosis group compared with a normal group. This could explain why the results of the IVIM parameters on hepatic steatosis were different according to study. We could suggest that the degree of hepatic steatosis in each study group influences the results of the affected IVIM parameters. In addition, another possible explanation of the increasing *f* value in pediatric hepatic steatosis is based on the histologic characteristics of hepatic steatosis of children. Pediatric hepatic steatosis has mainly portal inflammation with or without fibrosis, rather than ballooning degeneration or perisinusoidal fibrosis, which are more predominantly found in adult hepatic steatosis[12,24-26]. Perisinusoidal fibrosis and ballooning could lead to decreased microperfusion.

Instead, increased portal inflammation could lead to increased perfusion fraction due to increased blood volume within the liver. Further studies with pathologic correlation of various degrees and etiologies of hepatic steatosis are needed.

In hepatic fibrosis, our study demonstrated a negative correlation between fibrosis and D* in children after a Kasai operation for BA. In pediatric NAFLD, hepatic fibrosis showed a negative correlation with *f*[12]. Another study of adolescents and adults found that decreased microperfusion (D* and *f*) resulted in decreased ADC value in hepatic fibrosis, rather than alterations in molecular diffusion, after a Fontan operation[10]. However, there was no study that included pediatric patients for assessing hepatic fibrosis from BA. Previous studies of hepatic fibrosis from chronic liver disease in adults also demonstrated a negative correlation between hepatic fibrosis and D*[5,7,23], which are consistent with our study even though they focused on a different etiology of fibrosis. The reason for this inverse correlation could be because the increased protein in the extracellular matrix can lead to sinusoidal space obliteration and result in decreased hepatic microperfusion[5,7]. Yoon *et al*[7] mentioned that a decreased D* value could detect early changes in hepatic fibrosis better than ADC value. Even though there are similar characteristics of microperfusion change in pediatric BA (D* change), chronic liver disease in adults (D* change), and pediatric NAFLD (*f* change), additional studies on the effects of hepatic fibrosis from different etiologies on IVIM parameters are needed.

There were several limitations in this study. First, there were disproportional subject numbers in the groups, because it was a retrospective study. Second, we did not obtain the pathologic results of the liver in the children. However, we used the radiological and clinical data of the children to define three groups as reasonably as possible. Moreover, the obtained IVIM values of our normal group were similar to the normal values of a recently published meta-analysis study[27]. Third, several factors might influence liver perfusion, including inflammation and edema, were not analyzed. However, most of the children in our study were from an outpatient clinic and were undergoing routine follow-up, and we performed regression analysis including liver function tests to evaluate the effects of liver inflammation on IVIM parameters.

In conclusion, the liver IVIM DWI technique with multiple *b*-values was feasible and useful for evaluating hepatic steatosis and fibrosis in children using perfusion-related parameters of D* and *f*. There was a positive correlation between hepatic fat and blood volume, and a negative correlation between hepatic fibrosis and endovascular blood flow velocity, while diffusion-related parameters were not affected. Further study is needed to determine whether the IVIM parameters are affected by different histopathologies and etiologies of pediatric hepatic steatosis and fibrosis.

ARTICLE HIGHLIGHTS

Research background
Liver disease including steatosis and fibrosis is not uncommon in pediatric population. Quantitative and non-invasive examination of liver status is important in children because biopsy has a limited role from its sampling error and invasiveness.

Research motivation
Intravoxel incoherent motion (IVIM) magnetic resonance imaging (MRI) is an advanced diffusion-weighted imaging (DWI) technique. It can separate the perfusion and diffusion components from the conventional apparent diffusion coefficient (ADC) in the liver. Previous studies demonstrated IVIM values were more useful than ADC value for the evaluation of liver disease in adults, using the pure molecular diffusion (D), pseudo-diffusion (D*), and perfusion fracture (*f*) parameters. However, limited studies have assessed hepatic steatosis and fibrosis in children using IVIM. In addition, no study has focused on pediatric livers with enough *b*-values for IVIM.

Research objectives
The purpose of the study was to evaluate the usefulness of IVIM DWI technique with multiple *b*-values for evaluating hepatic steatosis and fibrosis in children and to know the correlation between IVIM parameters and the degree of hepatic steatosis and fibrosis measured with MRI in children.

Research methods
We retrospectively reviewed liver IVIM MRI of children (≤ 18 years) who performed MRI with 8 *b*-values under the suspicion of liver steatosis or fibrosis from 2013 to 2016. In addition to the conventional ADC value, perfusion related parameters (D*, *f*) and diffusion related parameter (D) were measured in the liver. Degree of hepatic steatosis was measured using MR proton density fat fracture (PDFF) and degree of hepatic fibrosis was measured using MR elastography (MRE). Perfusion and diffusion parameters were compared between normal, fatty liver, and fibrotic liver groups. Effects of hepatic steatosis and fibrosis on IVIM parameters were assessed.

Research results
Hepatic steatosis and fibrosis affected IVIM parameters differently in children. Perfusion related parameters (D*, *f*) were significantly lower in the fibrosis group compared with other groups. MR PDFF demonstrated a positive correlation with the *f*, representing blood volume. MRE value was negatively correlated with the D*, representing endovascular blood flow velocity. However, D and ADC value were not affected.

Research conclusions
Liver IVIM MRI with multiple *b*-values was useful for evaluating hepatic steatosis and fibrosis in children. Hepatic steatosis and fibrosis influenced perfusion and diffusion parameters differently. As increasing hepatic steatosis, blood volume was significantly increased. As increasing hepatic fibrosis, endovascular blood flow velocity was significantly decreased. However, true molecular diffusion and conventional ADC were not affected. Therefore, IVIM MRI can be a useful tool for evaluating hepatic steatosis and fibrosis in children.

Research perspectives
Perfusion related parameters from IVIM MRI can be used for the evaluation of hepatic steatosis and fibrosis in children. Further study is needed to determine whether the IVIM parameters are affected by different histopathologies and etiologies of pediatric hepatic steatosis and fibrosis.

REFERENCES

1   **Clemente MG**, Mandato C, Poeta M, Vajro P. Pediatric non-alcoholic fatty liver disease: Recent solutions, unresolved issues,

and future research directions. *World J Gastroenterol* 2016; **22**: 8078-8093 [PMID: 27688650 DOI: 10.3748/wjg.v22.i36.8078]

2   **Kasahara M**, Umeshita K, Sakamoto S, Fukuda A, Furukawa H, Uemoto S. Liver transplantation for biliary atresia: a systematic review. *Pediatr Surg Int* 2017; **33**: 1289-1295 [PMID: 28983725 DOI: 10.1007/s00383-017-4173-5]

3   **Sanchez-Valle A**, Kassira N, Varela VC, Radu SC, Paidas C, Kirby RS. Biliary Atresia: Epidemiology, Genetics, Clinical Update, and Public Health Perspective. *Adv Pediatr* 2017; **64**: 285-305 [PMID: 28688594 DOI: 10.1016/j.yapd.2017.03.012]

4   **Takahashi Y**, Fukusato T. Histopathology of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *World J Gastroenterol* 2014; **20**: 15539-15548 [PMID: 25400438 DOI: 10.3748/wjg.v20.i42.15539]

5   **Zhang J**, Guo Y, Tan X, Zheng Z, He M, Xu J, Mei Y, Zhang J, Zhao X, Wang C, Feng Y, Chan Q, Wu Y, Xu Y. MRI-based estimation of liver function by intravoxel incoherent motion diffusion-weighted imaging. *Magn Reson Imaging* 2016; **34**: 1220-1225 [PMID: 27262828 DOI: 10.1016/j.mri.2016.05.013]

6   **França M**, Martí-Bonmatí L, Alberich-Bayarri Á, Oliveira P, Guimaraes S, Oliveira J, Amorim J, Gonzalez JS, Vizcaíno JR, Miranda HP. Evaluation of fibrosis and inflammation in diffuse liver diseases using intravoxel incoherent motion diffusion-weighted MR imaging. *Abdom Radiol* (NY) 2017; **42**: 468-477 [PMID: 27638516 DOI: 10.1007/s00261-016-0899-0]

7   **Yoon JH**, Lee JM, Baek JH, Shin CI, Kiefer B, Han JK, Choi BI. Evaluation of hepatic fibrosis using intravoxel incoherent motion in diffusion-weighted liver MRI. *J Comput Assist Tomogr* 2014; **38**: 110-116 [PMID: 24378888 DOI: 10.1097/RCT.0b013e3182a589be]

8   **Murphy P**, Hooker J, Ang B, Wolfson T, Gamst A, Bydder M, Middleton M, Peterson M, Behling C, Loomba R, Sirlin C. Associations between histologic features of nonalcoholic fatty liver disease (NAFLD) and quantitative diffusion-weighted MRI measurements in adults. *J Magn Reson Imaging* 2015; **41**: 1629-1638 [PMID: 25256692 DOI: 10.1002/jmri.24755]

9   **Jiang H**, Chen J, Gao R, Huang Z, Wu M, Song B. Liver fibrosis staging with diffusion-weighted imaging: a systematic review and meta-analysis. *Abdom Radiol* (NY) 2017; **42**: 490-501 [PMID: 27678393 DOI: 10.1007/s00261-016-0913-6]

10 **Dijkstra H**, Wolff D, van Melle JP, Bartelds B, Willems TP, Oudkerk M, Hillege H, van den Berg AP, Ebels T, Berger RM, Sijens PE. Diminished liver microperfusion in Fontan patients: A biexponential DWI study. *PLoS One* 2017; **12**: e0173149 [PMID: 28257439 DOI: 10.1371/journal.pone.0173149]

11 **Lee JT**, Liau J, Murphy P, Schroeder ME, Sirlin CB, Bydder M. Cross-sectional investigation of correlation between hepatic steatosis and IVIM perfusion on MR imaging. *Magn Reson Imaging* 2012; **30**: 572-578 [PMID: 22285877 DOI: 10.1016/j.mri.2011.12.013]

12 **Manning P**, Murphy P, Wang K, Hooker J, Wolfson T, Middleton MS, Newton KP, Behling C, Awai HI, Durelle J, Paiz MN, Angeles JE, De La Pena D, McCutchan JA, Schwimmer JB, Sirlin CB. Liver histology and diffusion-weighted MRI in children with nonalcoholic fatty liver disease: A MAGNET study. *J Magn Reson Imaging* 2017; **46**: 1149-1158 [PMID: 28225568 DOI: 10.1002/jmri.25663]

13 **Shin HJ**, Kim HG, Kim MJ, Koh H, Kim HY, Roh YH, Lee MJ. Normal range of hepatic fat fraction on dual- and triple-echo fat quantification MR in children. *PLoS One* 2015; **10**: e0117480 [PMID: 25659155 DOI: 10.1371/journal.pone.0117480]

14 **Venkatesh SK**, Ehman RL. Magnetic resonance elastography of liver. *Magn Reson Imaging Clin N Am* 2014; **22**: 433-446 [PMID: 25086938 DOI: 10.1016/j.mric.2014.05.001]

15 **Serai SD**, Towbin AJ, Podberesky DJ. Pediatric liver MR elastography. *Dig Dis Sci* 2012; **57**: 2713-2719 [PMID: 22569825 DOI: 10.1007/s10620-012-2196-2]

16 **Towbin AJ**, Serai SD, Podberesky DJ. Magnetic resonance imaging of the pediatric liver: imaging of steatosis, iron deposition, and fibrosis. *Magn Reson Imaging Clin N Am* 2013; **21**: 669-680 [PMID: 24183519 DOI: 10.1016/j.mric.2013.05.001]

17 **Poynton CB**, Lee MM, Li Y, Laszik Z, Worters PW, Mackenzie JD, Courtier J. Intravoxel incoherent motion analysis of renal allograft diffusion with clinical and histopathological correlation in pediatric kidney transplant patients: A preliminary cross-sectional observational study. *Pediatr Transplant* 2017; **21** [PMID: 28653457 DOI: 10.1111/petr.12996]

18 **Kim JW**, Lee CH, Yoo KH, Je BK, Kiefer B, Park YS, Kim KA, Park CM. Intravoxel incoherent motion magnetic resonance imaging to predict vesicoureteral reflux in children with urinary tract infection. *Eur Radiol* 2016; **26**: 1670-1677 [PMID: 26373765 DOI: 10.1007/s00330-015-3986-7]

19 **ter Voert EE**, Delso G, Porto M, Huellner M, Veit-Haibach P. Intravoxel Incoherent Motion Protocol Evaluation and Data Quality in Normal and Malignant Liver Tissue and Comparison to the Literature. *Invest Radiol* 2016; **51**: 90-99 [PMID: 26405835 DOI: 10.1097/RLI.0000000000000207]

20 **Koh DM**, Collins DJ, Orton MR. Intravoxel incoherent motion in body diffusion-weighted MRI: reality and challenges. *AJR Am J Roentgenol* 2011; **196**: 1351-1361 [PMID: 21606299 DOI: 10.2214/AJR.10.5515]

21 **Guiu B**, Petit JM, Capitan V, Aho S, Masson D, Lefevre PH, Favelier S, Loffroy R, Vergès B, Hillon P, Krausé D, Cercueil JP. Intravoxel incoherent motion diffusion-weighted imaging in nonalcoholic fatty liver disease: a 3.0-T MR study. *Radiology* 2012; **265**: 96-103 [PMID: 22843768 DOI: 10.1148/radiol.12112478]

22 **Yu SM**, Ki SH, Baek HM. Nonalcoholic Fatty Liver Disease: Correlation of the Liver Parenchyma Fatty Acid with Intravoxel Incoherent Motion MR Imaging-An Experimental Study in a Rat Model. *PLoS One* 2015; **10**: e0139874 [PMID: 26460614 DOI: 10.1371/journal.pone.0139874]

23 **Sandrasegaran K**, Territo P, Elkady RM, Lin Y, Gasparis P, Borthakur G, Lin C. Does intravoxel incoherent motion reliably stage hepatic fibrosis, steatosis, and inflammation? *Abdom Radiol* (NY) 2018; **43**: 600-606 [PMID: 28828711 DOI: 10.1007/s00261-017-1263-8]

24 **Schwimmer JB**, Behling C, Newbury R, Deutsch R, Nievergelt C, Schork NJ, Lavine JE. Histopathology of pediatric nonalcoholic fatty liver disease. *Hepatology* 2005; **42**: 641-649 [PMID: 16116629 DOI: 10.1002/hep.20842]

25 **Brunt EM**, Kleiner DE, Wilson LA, Unalp A, Behling CE, Lavine JE, Neuschwander-Tetri BA; NASH Clinical Research NetworkA list of members of the Nonalcoholic Steatohepatitis Clinical Research Network can be found in the Appendix. Portal chronic inflammation in nonalcoholic fatty liver disease (NAFLD): a histologic marker of advanced NAFLD-Clinicopathologic correlations from the nonalcoholic steatohepatitis clinical research network. *Hepatology* 2009; **49**: 809-820 [PMID: 19142989 DOI: 10.1002/hep.22724]

26 **Dillman JR**, Trout AT, Costello EN, Serai SD, Bramlage KS, Kohli R, Xanthakos SA. Quantitative Liver MRI-Biopsy Correlation in Pediatric and Young Adult Patients With Nonalcoholic Fatty Liver Disease: Can One Be Used to Predict the Other? *AJR Am J Roentgenol* 2018; **210**: 166-174 [PMID: 29045180 DOI: 10.2214/AJR.17.18446]

27 **Li YT**, Cercueil JP, Yuan J, Chen W, Loffroy R, Wáng YX. Liver intravoxel incoherent motion (IVIM) magnetic resonance imaging: a comprehensive review of published data on normal values and applications for fibrosis and tumor evaluation. *Quant Imaging Med Surg* 2017; **7**: 59-78 [PMID: 28275560 DOI: 10.21037/qims.2017.02.03]

Clinical Trials Study

New fecal test for non-invasive *Helicobacter pylori* detection: A diagnostic accuracy study

Andrea Iannone, Floriana Giorgio, Francesco Russo, Giuseppe Riezzo, Bruna Girardi, Maria Pricci, Suetonia C Palmer, Michele Barone, Mariabeatrice Principi, Giovanni FM Strippoli, Alfredo Di Leo, Enzo Ierardi

Andrea Iannone, Michele Barone, Mariabeatrice Principi, Alfredo Di Leo, Enzo Ierardi, Section of Gastroenterology, Department of Emergency and Organ Transplantation, University of Bari, Bari 70124, Italy

Andrea Iannone, Department of Emergency and Organ Transplantation, University of Bari, Bari 70124, Italy

Floriana Giorgio, Bruna Girardi, Maria Pricci, THD SpA, Correggio (Reggio Emilia) 42015, Italy

Francesco Russo, Giuseppe Riezzo, National Institute of Gastroenterology, "S De Bellis" Research Hospital, CastellanaGrotte (Bari) 70013, Italy

Suetonia C Palmer, Department of Medicine, University of Otago Christchurch, Christchurch 8011, New Zealand

Giovanni FM Strippoli, Diaverum Academy, Lund 22229, Sweden

Giovanni FM Strippoli, Diaverum Medical Scientific Office, Lund 22229, Sweden

Giovanni FM Strippoli, Sydney School of Public Health, University of Sydney, Sydney NSW-2000, Australia

Giovanni FM Strippoli, Section of Nephrology, Department of Emergency and Organ Transplantation, University of Bari, Bari 70124, Italy

ORCID number: Andrea Iannone (0000-0002-5468-9515); Floriana Giorgio (0000-0001-5209-1256); Francesco Russo (0000-0003-0538-6072); Giuseppe Riezzo (0000-0002-1800-4830); Bruna Girardi (0000-0003-3848-7996); Maria Pricci (0000-0002-2158-6392); Suetonia C Palmer (0000-0001-7765-5871); Michele Barone (0000-0001-8284-5127); Mariabeatrice Principi (0000-0003-0545-5656); Giovanni FM Strippoli (0000-0002-6936-0616); Alfredo Di Leo (0000-0003-2026-1200); Enzo Ierardi (0000-0001-7275-5080).

Author contributions: Iannone A, Di Leo A and Ierardi E designed the research; Iannone A, Giorgio F, Russo F, Riezzo G, Girardi B, Pricci M and Principi M performed the research;

Giorgio F, Russo F, Riezzo G, Girardi B and Pricci M collected the data; Iannone A analyzed the data; Iannone A, Palmer SC, Strippoli GF and Ierardi E wrote the paper; Palmer SC, Barone M, Principi M, Strippoli GF, Di Leo A and Ierardi E critically revised the manuscript for important intellectual content.

Abstract

AIM

To assess the diagnostic accuracy of a new fecal test for detecting *Helicobacter pylori* (*H. pylori*), using ^{13}C-urea breath test as the reference standard, and explore bacterial antibiotic resistance.

METHODS

We conducted a prospective two-center diagnostic test accuracy study. We enrolled consecutive people ≥ 18 years without previous diagnosis of *H. pylori* infection, referred for dyspepsia between February and October 2017. At enrollment, all participants underwent ^{13}C-urea breath test. Participants aged over 50 years were scheduled to undergo upper endoscopy with histology. Participants collected stool samples 1-3 d after enrollment for a new fecal investigation (THD fecal test). The detection of bacterial 23S rRNA subunit gene indicated *H. pylori* infection. We also used the index diagnostic test to examine mutations conferring resistance to clarithromycin and levofloxacin. Independent investigators analyzed index test and reference test standard results blinded to the other test findings. We estimated sensitivity, specificity, positive (PPV) and negative (NPV) predictive value, diagnostic accuracy, positive and negative likelihood ratio (LR), together with 95% confidence intervals (CI).

RESULTS

We enrolled 294 consecutive participants (age: Median 37.0 years, IQR: 29.0-46.0 years; men: 39.8%). Ninety-five (32.3%) participants had a positive ^{13}C-urea breath test. Twenty-three (7.8%) participants underwent upper endoscopy with histology, with a full concordance between ^{13}C-urea breath test and histology in detecting *H. pylori* infection. Four (1.4%) out of the 294 participants withdrew from the study after the enrollment visit and did not undergo THD fecal testing. In the 290 participants who completed the study, the THD fecal test sensitivity was 90.2% (CI: 84.2%-96.3%), specificity 98.5% (CI:96.8%-100%), PPV 96.5% (CI: 92.6%-100%), NPV 95.6% (CI: 92.8%-98.4%), accuracy 95.9% (CI: 93.6%-98.2%), positive LR 59.5(CI: 19.3-183.4), negative LR 0.10 (CI: 0.05-0.18). Out of 83 infected participants identified with the THD fecal test, 34 (41.0%) had bacterial genotypic changes consistent with antibiotic-resistant *H. pylori* infection. Of these, 27 (32.5%) had bacterial strains resistant to clarithromycin, 3 (3.6%) to levofloxacin, and 4 (4.8%) to both antibiotics.

CONCLUSION

The THD fecal test has high performance for the non-

invasive diagnosis of *H. pylori* infection while additionally enabling the assessment of bacterial antibiotic resistances.

Core tip: Existing studies on molecular tests for *Helicobacter pylori* (*H. pylori*) detection in stools show suboptimal quality. The THD fecal test is a newer method to detect bacterial DNA and mutations conferring antibiotic resistance. In this diagnostic test accuracy study involving unselected consecutive participants and blinded outcome assessment, we evaluated the diagnostic accuracy of the THD fecal test for detecting *H. pylori*, using the ^{13}C-urea breath test as the reference standard. We found that the THD fecal test has high performance for the non-invasive diagnosis of *H. pylori* infection while additionally enabling the assessment of bacterial antibiotic resistances.

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection occurs among 48.5% of the general population worldwide, with high geographic variability[1]. It is the leading cause of chronic/atrophic gastritis, peptic ulcer, gastric lymphoma, gastric carcinoma, and some extra-gastric disorders[2-4]. Diagnostic approaches for *H. pylori* infection include invasive and non-invasive testing[4-6]. The ^{13}C-urea breath test is established as a highly sensitive and specific test (96% sensitivity and 93% specificity) for the non-invasive diagnosis ofinfection[4,7]. A stool-based monoclonal antigen test has low acceptability in some contexts and needs local validation, despite high accuracy[4,8]. Invasive tests require upper endoscopy, limiting their application based on local practices and policies and to patients who have alarm symptoms[4,5,9]. Culture with antibiogram is only recommended after repeated treatment failures[4-6] due to its high false negative rate[10,11].

Molecular testing is a promising approach for diagnosing *H. pylori* infection and has the added advantage of identifying bacterial DNA mutations associated with antibiotic resistance. Molecular tests on gastric biopsy are commonly used only for research purposes due to

the need for an invasive endoscopic procedure[12].Thus, the application of these tests on fecal samples is gaining interest. A recent meta-analysis identified the bacterial 23S ribosomal RNA subunit gene as the most accurate marker for diagnosis of infection using molecular tests on stool samples, with 82% (95%CI: 77%-86%) sensitivity and 99%(95%CI: 98%-100%) specificity[13]. The consistency of these results is limited by the inclusion of studies of suboptimal quality resulting from bias in participant selection and lack of blinded outcome assessment[14-19].

We aimed to assess the diagnostic accuracy of a new molecular test, theTHD fecal test[20], for the non-invasive detection of *H. pylori* DNA, using the ^{13}C-urea breath test as the reference standard. We estimated the point prevalence of *H. pylori* DNA point mutations conferring resistance to clarithromycin and levofloxacin.

MATERIALS AND METHODS

This study was conducted according to the Standards for Reporting of Diagnostic Accuracy (STARD) statement[21].

Study design

We performed a two-center cross-sectional study with prospective data collection. We included consecutive participantsexperiencing dyspeptic symptoms and without previous diagnosis of *H. pylori* infection, who were referred for diagnostic evaluation to the Gastroenterology Unit, University of Bari (Italy) or the National Institute of Gastroenterology "Saverio De Bellis", CastellanaGrotte, Bari (Italy) between February and October 2017.

Participants were eligible if they were aged 18 years or older and had experienced dyspeptic symptoms, defined as the presence of one or more of: post-prandial fullness, early satiation, epigastric pain and epigastric burning for at least one (post-prandial fullness and early satiation) or three (epigastric pain and epigastric burning) days per week in the last three months with symptoms onset at least six months previously[22]. Exclusion criteria were treatment with proton pump inhibitors or 2-histamine receptor antagonists in the previous two weeks as well as use of antibiotics or bismuth salts in the previous four weeks, as these medications may increase false negative results of invasive and non-invasive current diagnostic tests for *H. pylori* infection by reducing the bacterial load[23-25]. Additional exclusion criteria were previous diagnosis of *H. pylori* infection and presence of chronic diarrhea, which can limit the accurate collection of stool samples for the THD fecal test. Potential participants were also excluded if they had alarm symptoms, including weight loss, dysphagia, gastrointestinal bleeding, an abdominal mass or iron deficiency anemia, which are an indication to perform upper endoscopy as a first-line diagnostic approach[4,5,9].

At the enrollment visit, eligible participants underwent

the ^{13}C-urea breath test (the reference standard) for the non-invasive investigation of *H. pylori* infection. According to current Italian Clinical guideline recommendations[5], participants older than 50 years were scheduled to undergo upper endoscopy with biopsy sampling for histology within one week. All participants were asked to provide stool samples collected 1 to 3 d after enrollment, using the THD device. These samples were used for the THD fecal test (index test). Independent investigators analyzed the index test and reference standard test results blinded to the other test findings, participants' information and histology results. Pathologists performing histology examination were unaware of the results of the other two tests.

Thestudy was performed in agreement with the ethical guidelines of the Declaration of Helsinki and the protocol was approved by the local Ethics Committee (Ospedale Consorziale Policlinico, Bari, protocol number 74413). All participants gave written informed consent before inclusion in the study.

THD fecal test (index test)

The technical details of *H. pylori* DNA extraction and analysis are reported in Appendix 1.

Within three days after the enrollment visit, participants collected and stored a stool sample using the THD fecal test equipment (THD Spa, Correggio, Reggio Emilia, Italy), which allows for obtaining an adequate stool-derived product to extract *H. pylori* DNA.

We pre-specified THD fecal test positivity as the identification of the *H. pylori* bacterial gene encoding the 23S ribosomal RNA subunit in the stool-derived product[20]. The detection of specific bacterial DNA point mutations indicated *H. pylori* resistance to clarithromycin and/or levofloxacin. In brief, we assessed A2142C, A2142G and A2143G point mutations in the 23S rRNA subunit gene for clarithromycin resistance, and C261A, C261G, G271A, A272G, G271T and A270T point mutations in the A-subunit of gyrase gene for levofloxacin resistance.

^{13}C-urea breath test (reference standard) and upper endoscopy

The technical details of the ^{13}C-urea breath test are reported in Appendix 2.

At the enrollment visit, all participants underwent ^{13}C-urea breath testing after overnight fasting. We used this test as the reference standard due to the non-invasiveness, high diagnostic accuracy (sensitivity of 96% and specificity of 93%), and consumer acceptance[4,7]. We pre-specified a ^{13}C-urea breath test positivity for a difference between the baseline and 30 min breath sample that exceeded 4 parts per 1000 of ^{13}carbon dioxide ($^{13}CO_2$)[26,27].

Participants older than 50 years were scheduled to undergo upper endoscopy with biopsy sampling for histology, according to current Italian guidelines[5]. Among these participants, a minimum of two biopsy

Table 1 Characteristics of the participants included in the study *n* (%)

Characteristic	Value (*n* = 294)
Age, median (IQR), yr	37.0 (29.0-46.0)
Female sex, number	177 (60.2)
Helicobacter pylori infection, number	95 (32.3)[1]
Upper endoscopy with histology, number	23 (7.8)[2]
Dyspeptic symptoms, number	
Post-prandial fullness	115 (39.1)
Early satiation	50 (17.0)
Epigastric pain	130 (44.2)
Epigastric burning	170 (57.8)
Concomitant diseases, number	
Cardiovascular disease	8 (2.7)
Chronic kidney disease/dialysis	0 (0)
Chronic liver disease/cirrhosis	3 (1.0)
Other chronic diseases[3]	10 (3.4)

[1]Diagnosed with the ^{13}C-urea breath test (reference standard test); [2]There were 40 participants older than 50 years, but 17 refused to undergo upper endoscopy; [3]Including asthma (8 participants), spasmophilia (1 participant), multiple sclerosis (1 participant). IQR: Interquartile range.

samples from the gastric antrum (greater and lesser curvature, 3 cm proximal to the pyloric region) and two from the middle of the gastric body were collected for histologic examination[4].

Statistical analysis

We assessed the normal distribution of continuous variables using the Shapiro-Wilk test and expressed them as the mean and standard deviation (SD) or median and interquartile range (IQR). We expressed categorical variables asa percentage.

The results of the ^{13}C-urea breath test served as the reference standard for assessing the diagnostic accuracy of the THD fecal test in detecting H. *pylori* infection. We calculated sensitivity, specificity, negative and positive predictive values, and diagnostic accuracy for the THD fecal test together with 95% confidence intervals (CI), according to standard definitions. Since the prevalence of the condition in the enrolled population influences sensitivity, specificity, and predictive values, we also calculated the positive and negative likelihood ratios. To identify the impact of our findings on clinical decision-making, we calculated the post-test probability after positive and negative results onthe THD fecal test for populations with different pre-test probabilities of *H. pylori* infection based on likelihood ratios.

We pre-planned to handle uninterpretable and missing index test or reference standard findings with both the "complete case" approach and "best-worst case" imputation, to avoid overestimation of diagnostic accuracy parameters[28]. In detail, these results were removed for the "complete case" analysis, while they were considered as false-negatives and false-positivesfor the "worst case" analysis or as true-negatives and true-positives for the "best-case" analysis.

We estimatedthe point prevalence of *H. pylori* resis-

tance to clarithromycin and levofloxacinas the number of participant with the specific antibiotic resistance divided by the total number of participants diagnosed with *H. pylori* infection at THD fecal test.

We assumed a 94% sensitivity and 97% specificity for the THD fecal test, based on monoclonal stool antigen test accuracy parameters for the lack of high-quality evidence on molecular fecal tests[8]. Assuming a *H. pylori* infection prevalence of 34.3%[1], a marginal error of 0.05, and a drop-out of 10%, we calculated a sample size of at least 280 participants, to provide 80% power with an α of 0.05 to detect the pre-specified sensitivity and specificity values for the THD fecal test[29].

We used Statistical Analysis Software (SAS Institute Inc., Cary, NC, United States) 9.4 for all the analyses.

RESULTS

Characteristics of study population

During the enrollment period, 305 consecutive participants were eligible for inclusion in the study. Of these, 11 were excluded for preference not to participate.Four out of the 294 participants withdrew from the study after the enrollment visit did not undergo THD fecal testing (Figure 1).

Table 1 describes the characteristics of the 294 participants included in the study. The median age was 37.0 years (IQR: 29.0 to 46.0 years). There were 177 (60.2%) women. The ^{13}C-urea breath test (reference standard test) was positive in 95 (32.3%) participants. Forty (13.6%) participants were older than 50 years and were scheduled to undergo upper endoscopy with histology. Of these, 23 (7.8%) participants, 7 with and 16 without *H. pylori* infection, agreed to undergo this examination. There was full concordance between the ^{13}C-urea breath test and histology in detecting *H. pylori* infection in these participants. With regard to dyspeptic symptoms reported at enrollment visit, 115 (39.1%) participants experienced post-prandial fullness, 50 (17.0%) early satiation, 130 (44.2%) epigastric pain, and 170 (57.8%) epigastric burning. There were no reported adverse events during performance of the index or reference standard test.

THD fecal test diagnostic performance

The amplification curves of *H. pylori* DNA sequences from the real time polymerase chain reaction are shown in Appendix 3.

Direct comparisons between the ^{13}C-urea breath test and THD fecal test results for detecting *H. pylori* infection are reported in Table 2.

The diagnostic accuracy parameters of the THD fecal test for detecting *H. pylori* infection, using ^{13}C-urea breath test as the reference standard, are shown in Table 3. In the "complete-case" analysis, including the 290 participants who completed the study, the THD
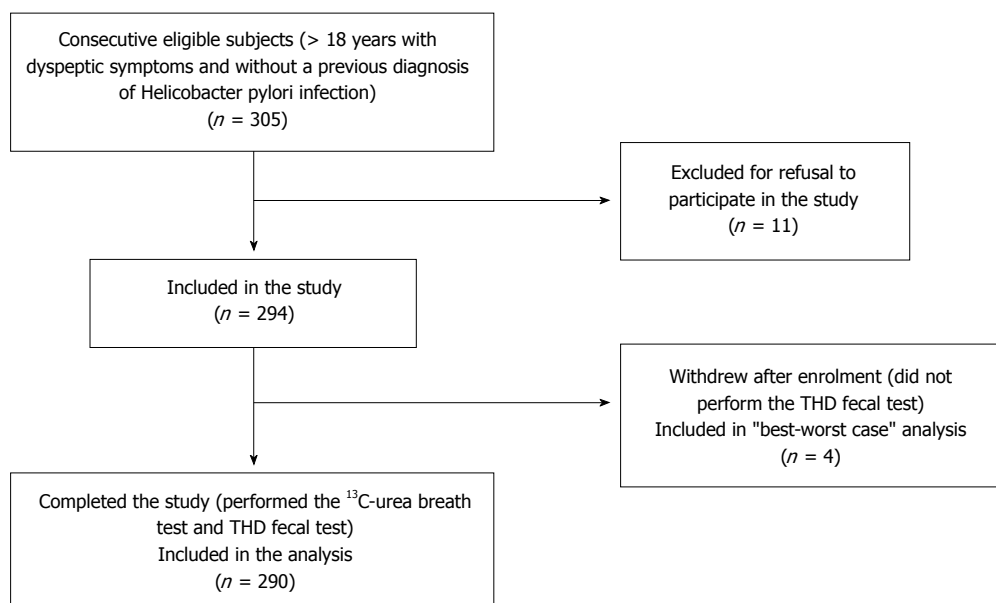
Figure 1 Flow diagram of participant recruitment in the study.

fecal test had a sensitivity of 90.2% (CI: 84.2% to 96.3%), specificity of 98.5% (CI: 96.8% to 100%), positive predictive value of 96.5% (CI: 92.6% to 100%), and negative predictive value of 95.6% (CI: 92.8% to 98.4%). The accuracy of the index test in correctly identifying participants with and without *H. pylori* infection was 95.9% (CI: 93.6% to 98.2%). The THD fecal test positive likelihood ratio was 59.5 (CI: 19.3 to 183.4), and the negative likelihood ratio was 0.10 (CI: 0.05 to 0.18). In the "best-worst case" analysis, including all 294 participants enrolled in the study, there were small changes in THD fecal test diagnostic accuracy parameters (Table 3).

Figure 2 displays the estimation of the probability of *H. pylori* infection after positive and negative THD fecal test results for populations with different prevalence of disease, based on likelihood ratios calculated from our data. As shown, the probability of infection after a positive index test finding is higher than 90% in populations with a disease prevalence ≥ 20%. The probability of infection after a negative index test finding is lower than 10% and 20% in populations with disease prevalence ≤ 50% and ≤ 70%, respectively.

H. pylori resistance rates to clarithromycin and levofloxacin

Table 4 summarizes the *H. pylori* resistance rates to clarithromycin and levofloxacin in the 83 *H. pylori* infected participants identified with the THD fecal test. Thirty-four (41.0%) participants had bacterial genotypic changes consistent with antibiotic-resistant *H. pylori* infection. Of these, 27 (32.5%) had bacterial strains resistant to clarithromycin, 3 (3.6%) to levofloxacin, and 4 (4.8%) to both antibiotics. The overall resistance rates were 37.3% (31 participants) to clarithromycin

and 8.4% (7 participants) to levofloxacin.

DISCUSSION

Main findings

In this diagnostic test accuracy study involving unselected consecutive participants and blinded outcome assessment, we observed that the non-invasive THD fecal test has high performance for the diagnosis of *H. pylori* infection among patients with dyspeptic symptoms. This test showed a 90.2% sensitivity and 98.5% specificity, with a diagnostic accuracy of 95.9%. Considering the worldwide prevalence of *H. pylori* infection, ranging from 70% in Africa to 24% in Oceania[1], there was > 90% post-test probability of bacterial infection after a positive THD fecal test result and < 20% probability after a negative finding. In Western Europe, the prevalence of infection is around 34%[1], leading to post-test probabilities > 95% and < 5% after a positive and negative test, respectively. The THD fecal test identified *H. pylori* resistance rates of 32.5% to clarithromycin, 3.6% to levofloxacin, and 4.8% to both antibiotics.

Comparison with existing knowledge

A recent meta-analysis found that the 23S rRNA subunit gene is the most accurate marker for detecting *H. pylori* in stools using molecular analyses based on real time polymerase chain reaction. The pooled analysis of six diagnostic accuracy studies showed estimated sensitivity of 82% (95%CI: 77% to 86%) and specificity of 99% (95%CI: 98% to 100%) for the bacterial 23S rRNA subunit gene[13]. Comparing our results with findings from existing primary studies[14-19] included in this meta-analysis is difficult, mainly due to

Table 2 Direct comparisons between the ¹³C-urea breath test and THD fecal test results for detecting participants with *Helicobacter pylori* infection

	¹³C-urea breath test	
	Positive	Negative
THD fecal test		
Positive	83	3
Negative	9	195
Missing[1]	3	1
Total	95	199

[1]Four participants withdrew from the study after enrollment, thus they underwent the ¹³C-urea breath test but not the THD fecal test.
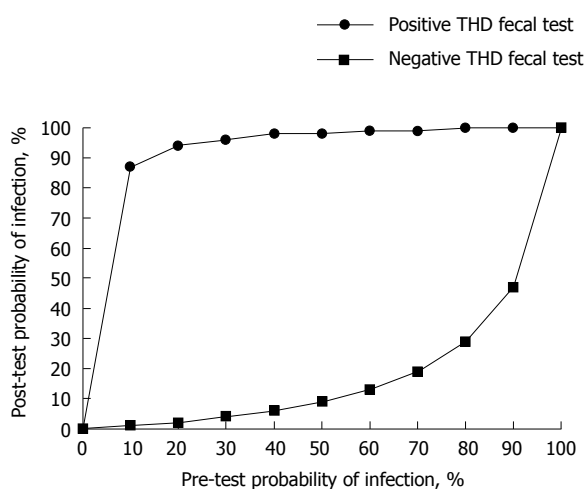

Figure 2 Post-test probability after positive and negative results on the THD fecal test for populations with different pre-test probabilities of *Helicobacter pylori* infection, based on likelihood ratios.

limitations in methodological reporting in these studies and differences in the selection of population and the reference standard used. Four studies[14,15,17,19] included children (age < 18 years), one[18] did not specify the participants' age, and one[16] included adults (range 20 to 81 years), with an estimated disease prevalence ranging between 21% and 81% among studies. The reference standard was monoclonal stool antigen test in three studies[16-18], a combination of histology, rapid urease test, and culture in two studies[14,19], and a combination of histology, culture, ¹³C-urea breath test and monoclonal stool antigen test in one study[15]. In two studies[14,19], consecutive participants were enrolled, while in the remaining four[15-18], a convenience sample was selected. No authors provided the sample size estimation. There was no reported blinding of the assessment of the index and reference standard test findings, except for pathologists analyzing gastric biopsy samples in one study[15]. Thus, the use of convenience samples and lack of blinding may have led to an overestimation of the diagnostic accuracy of these molecular tests for the non-invasive diagnosis of *H. pylori* infection. Despite the selection of consecutive participants and the blinded assessment of the index

and reference standard results in our study, we found greater test sensitivity and similar high specificity compared to the pooled estimate of earlier studies. This may be due to the high performance of the THD device for obtaining adequate stool sample-derived product to extract *H. pylori* DNA. Our sensitivity estimate is concordant with that reported in a recent publication[30], although this last study is burdened by the same methodological limitations and risk of bias as previous researches.

Our *H. pylori* resistance rates to clarithromycin and levofloxacinwere consistent with those reported in previous epidemiologic studies from the same geographic area[31]. Molecular analyses performed on stool samples showed bacterial resistance rates of 36%-41% to clarithromycin[16,30], in agreement with our estimate of 37.3%. There is limited evidence on the use of molecular fecal tests to detect *H. pylori* resistance to levofloxacin. Recently, the molecular analysis "Genotype HelicoDR assay" (Hain Lifescience GmbH, Nehren, Germany) has been applied to fecal samples for detecting clarithromycin and fluoroquinolone resistance using molecular analysis on gastric biopsy, with proven high accuracy, as the reference standard[32]. This test identified a resistance rate to fluoroquinolone of 13% in stools, which was somewhat higher than the present study (8.4%). However, there was low agreement between stool and biopsy findings for resistance to both clarithromycin (53%) and fluoroquinolone (35%), indicating a poor performance of this test for the non-invasive assessment of *H. pylori* resistances to antibiotics. By contrast, the THD fecal test has shown high concordance with real time polymerase chain reaction-based molecular analysis on gastric tissue for detecting bacterial resistance to clarithromycin[20].

Strengths and limitations
This study investigated the diagnostic accuracy parameters of a novel molecular tool (THD fecal test) for the non-invasive diagnosis of *H. pylori* infection in consecutive participants with dyspepsia. The strengths of the study include an *a priori* sample size, prospectively enrolled consecutive participants, and blinded assessment of the index and reference standard results to increase the certainty of the findings. Our study also assessed the feasibility of detecting bacterial resistance to clarithromycin and levofloxacin, using a non-invasive approach.

Our study has some limitations that should be considered when interpreting the results. First, we diagnosed *H. pylori* infection using a single non-invasive test, while a second confirmation test (histology) was performed only in the subgroup of participants older than 50 years. However, the ¹³C-urea breath test is recommended as the gold-standard non-invasive diagnostic approach for detecting this bacterial infection, with 96% sensitivity and 93% specificity[4,7]. We also

Table 3 Diagnostic accuracy parameters of the THD fecal test for detecting *Helicobacter pylori* infection, using ^{13}C-urea breath test as the reference standard

Parameter	Complete-case analysis (n = 290)	Best-case analysis (n = 294)[1]	Worst-case analysis (n = 294)[1]
Sensitivity, % (95%CI)	90.2 (84.2 to 96.3)	90.5 (84.6 to 96.4)	87.4 (80.7 to 94.1)
Specificity, % (95%CI)	98.5 (96.8 to 100)	98.5 (96.8 to 100)	98.0 (96.0 to 99.9)
PPV, % (95%CI)	96.5 (92.6 to 100)	96.6 (92.9 to 100)	95.4 (91.0 to 99.8)
NPV, % (95%CI)	95.6 (92.8 to 98.4)	95.6 (92.8 to 98.4)	94.2 (91.0 to 97.4)
Accuracy, % (95%CI)	95.9 (93.6 to 98.2)	95.9 (93.7 to 98.2)	94.6 (92.0 to 97.2)
Positive LR, estimate (95%CI)	59.5 (19.3 to 183.4)	60.0 (19.5 to 185.0)	43.5 (16.4 to 115.0)
Negative LR, estimate (95%CI)	0.10 (0.05 to 0.18)	0.10 (0.05 to 0.18)	0.13 (0.08 to 0.22)

[1]The analysis includes four participants with missing data for the THD fecal test. PPV: Positive predictive value; NPV: Negative predictive value; LR: Likelihood ratio; CI: Confidence interval.

Table 4 *Helicobacter pylori* resistance rates to clarithromycin and levofloxacin in the 83 infected participants identified with the THD fecal test *n* (%)

	Clarithromycin		Total
	Susceptible	Resistant	
Levofloxacin			
Susceptible	49 (59.1)	27 (32.5)	76 (91.6)
Resistant	3 (3.6)	4 (4.8)	7 (8.4)
Total	52 (62.7)	31 (37.3)	83 (100)

found complete agreement between ^{13}C-urea breath test and histology in participants older than 50 years. Moreover, current international guidelines[4,5,9] recommend a test-and-treat strategy for *H. pylori* infection in young (≤ 50 years in Italy) dyspeptic people without alarm symptoms to avoid the costs, inconvenience and discomfort of endoscopy. Thus, our approach reflects the current clinical practice and the most appropriate diagnostic strategy for this population. Second, we did not perform molecular analysis of bacterium resistance to clarithromycin and levofloxacin on gastric biopsy samples to confirm the results obtained on stool samples. Thus, we did not calculate diagnostic accuracy parameters of the THD fecal test for detecting the bacterial resistance to these antibiotics. However, we have previously demonstrated full concordance between THD fecal test and molecular analysis on gastric tissue findings for detecting *H. pylori* resistance to clarithromycin[20].

Implications for practice and research / conclusions

In conclusion, our results indicate that the THD fecal test has high diagnostic performance for non-invasive detection of *H. pylori* infection in patients with dyspeptic symptoms while enabling identification of bacterium resistance to clarithromycin and levofloxacin. The THD fecal test may assist in the conduct of randomized trials to evaluate the benefits and harms of tailored eradication strategies in first-line. On these bases, the THD fecal test may inform clinical decision-making and guide individualized treatments for *H. pylori* infection.

ARTICLE HIGHLIGHTS

Research background

Diagnostic approaches for *Helicobacter pylori* (*H. pylori*) infection include invasive and non-invasive testing. The non-invasive ^{13}C-urea breath test and stool monoclonal antigen test have high accuracy for diagnosing the infection, although the stool test has low acceptability in some contexts and needs local validation. The need for upper endoscopy is a limitation to the use of invasive tests. Molecular tests are promising approaches for diagnosing *H. pylori* infection, due to the added advantage of identifying bacterial DNA mutations associated with antibiotic resistance.

Research motivation

The application of molecular diagnostic tests on gastric biopsy samples is limited by the need for invasive endoscopic procedure. Thus, the non-invasive application of these tests on fecal samples is gaining increasing interest. An accurate non-invasive molecular test may guide first-line eradicating treatments with the potential advantages of increasing bacterial eradication rates and reducing the development of *H. pylori* resistance to antibiotics. However, existing studies on molecular tests for *H. pylori* detection in stools show suboptimal quality.

Research objectives

We aimed to assess the accuracy of a new non-invasive molecular test, the THD fecal test, for the diagnosis of *H. pylori* infection, using ^{13}C-urea breath test as the reference standard. Additionally, we estimated the point prevalence of *H. pylori* DNA mutations conferring resistance to clarithromycin and levofloxacin.

Research methods

We conducted a prospective two-center diagnostic test accuracy study. We enrolled consecutive people ≥ 18 years old without previous diagnosis of *H. pylori* infection, referred for dyspepsia between February and October 2017. At enrollment, all participants underwent ^{13}C-urea breath test. Participants aged over 50 years were scheduled to undergo upper endoscopy with histology. Participants collected stool samples 1-3 d after enrollment for the THD fecal test. The detection of bacterial 23S rRNA subunit gene indicated *H. pylori* infection. We also used the index diagnostic test to examine mutations conferring resistance to clarithromycin and levofloxacin. Independent investigators analyzed the index test and reference standard test results blinded to the other test findings, participants' information and histology results. We estimated diagnostic accuracy parameters, together with their 95% confidence intervals. The novelty of our research methods included an *a priori* sample size, a prospective enrollment of consecutive participants, and the blinding of outcome assessors. This approach increased the certainty of our findings.

Research results

Out of 294 participants, 95 (32.3%) had a positive ^{13}C-urea breath test. Four (1.4%) participants withdrew from the study after the enrollment visit. In the

290 participants who completed the study, the THD fecal test sensitivity was 90.2% (CI: 84.2%-96.3%), specificity 98.5% (CI: 96.8%-100%), positive predictive value 96.5% (CI: 92.6%-100%), negative predictive value 95.6% (CI: 92.8%-98.4%), accuracy 95.9% (CI: 93.6%-98.2%), positive likelihood ratio 59.5 (CI: 19.3-183.4), negative likelihood ratio 0.10 (CI: 0.05-0.18). Out of 83 *H. pylori* infected participants identified with the THD fecal test, 27 (32.5%) had bacterial strains resistant to clarithromycin, 3 (3.6%) to levofloxacin, and 4 (4.8%) to both antibiotics.

Research conclusions

Our results indicate that the THD fecal test has high diagnostic accuracy for the non-invasive diagnosis of *H. pylori* infection in patients with dyspeptic symptoms, while enabling identification of bacterium resistance to clarithromycin and levofloxacin. The certainty of our findings is based on the rigorous methodological approach used in the assessment of the THD fecal test diagnostic performance. THD fecal testing may inform clinical decision-making and guide individualized therapies to eradicate *H. pylori* infection.

Research perspectives

The spread of *H. pylori* resistance to antibiotics has prompted the investigation of the efficacy of antibiotic susceptibility-guided therapies. THD fecal testing may assist in the conduct of randomized trials to evaluate the benefits and harms of tailored eradication strategies in first-line.

REFERENCES

1   **Hooi JKY**, Lai WY, Ng WK, Suen MMY, Underwood FE, Tanyingoh D, Malfertheiner P, Graham DY, Wong VWS, Wu JCY, Chan FKL, Sung JJY, Kaplan GG, Ng SC. Global Prevalence of Helicobacter pylori Infection: Systematic Review and Meta-Analysis. *Gastroenterology* 2017; **153**: 420-429 [PMID: 28456631 DOI: 10.1053/j.gastro.2017.04.022]

2   **Suerbaum S**, Michetti P. Helicobacter pylori infection. *N Engl J Med* 2002; **347**: 1175-1186 [PMID: 12374879 DOI: 10.1056/NEJMra020542]

3   **Graham DY**, Lew GM, Klein PD, Evans DG, Evans DJ Jr, Saeed ZA, Malaty HM. Effect of treatment of Helicobacter pylori infection on the long-term recurrence of gastric or duodenal ulcer. A randomized, controlled study. *Ann Intern Med* 1992; **116**: 705-708 [PMID: 1558340]

4   **Malfertheiner P**, Megraud F, O'Morain CA, Gisbert JP, Kuipers EJ, Axon AT, Bazzoli F, Gasbarrini A, Atherton J, Graham DY, Hunt R, Moayyedi P, Rokkas T, Rugge M, Selgrad M, Suerbaum S, Sugano K, El-Omar EM; European Helicobacter and Microbiota Study Group and Consensus panel. Management of Helicobacter pylori infection-the Maastricht V/Florence Consensus Report. *Gut* 2017; **66**: 6-30 [PMID: 27707777 DOI: 10.1136/gutjnl-2016-312288]

5   **Zagari RM**, Romano M, Ojetti V, Stockbrugger R, Gullini S, Annibale B, Farinati F, Ierardi E, Maconi G, Rugge M, Calabrese C, Di Mario F, Luzza F, Pretolani S, Savio A, Gasbarrini G, Caselli M. Guidelines for the management of Helicobacter pylori infection in Italy: The III Working Group Consensus Report 2015. *Dig Liver Dis* 2015; **47**: 903-912 [PMID: 26253555 DOI: 10.1016/j.dld.2015.06.010]

6   **Fallone CA**, Chiba N, van Zanten SV, Fischbach L, Gisbert JP, Hunt RH, Jones NL, Render C, Leontiadis GI, Moayyedi P, Marshall JK. The Toronto Consensus for the Treatment of Helicobacter pylori Infection in Adults. *Gastroenterology* 2016; **151**: 51-69.e14 [PMID: 27102658 DOI: 10.1053/j.gastro.2016.04.006]

7   **Ferwana M**, Abdulmajeed I, Alhajiahmed A, Madani W, Firwana B, Hasan R, Altayar O, Limburg PJ, Murad MH, Knawy B. Accuracy of urea breath test in Helicobacter pylori infection: meta-analysis. *World J Gastroenterol* 2015; **21**: 1305-1314 [PMID: 25632206 DOI: 10.3748/wjg.v21.i4.1305]

8   **Gisbert JP**, de la Morena F, Abraira V. Accuracy of monoclonal stool antigen test for the diagnosis of H. pylori infection: a systematic review and meta-analysis. *Am J Gastroenterol* 2006; **101**: 1921-1930 [PMID: 16780557 DOI: 10.1111/j.1572-0241.2006.00668.x]

9   **Chey WD**, Leontiadis GI, Howden CW, Moss SF. ACG Clinical Guideline: Treatment of Helicobacter pylori Infection. *Am J Gastroenterol* 2017; **112**: 212-239 [PMID: 28071659 DOI: 10.1038/ajg.2016.563]

10 **Ierardi E**, Giorgio F, Iannone A, Losurdo G, Principi M, Barone M, Pisani A, Di Leo A. Noninvasive molecular analysis of Helicobacter pylori: Is it time for tailored first-line therapy? *World J Gastroenterol* 2017; **23**: 2453-2458 [PMID: 28465629 DOI: 10.3748/wjg.v23.i14.2453]

11 **Kjøller M**, Fischer A, Justesen T. Transport conditions and number of biopsies necessary for culture of Helicobacter pylori. *Eur J Clin Microbiol Infect Dis* 1991; **10**: 166-167 [PMID: 2060517]

12 **De Francesco V**, Margiotta M, Zullo A, Hassan C, Troiani L, Burattini O, Stella F, Di Leo A, Russo F, Marangi S, Monno R, Stoppino V, Morini S, Panella C, Ierardi E. Clarithromycin-resistant genotypes and eradication of Helicobacter pylori. *Ann Intern Med* 2006; **144**: 94-100 [PMID: 16418408]

13 **Khadangi F**, Yassi M, Kerachian MA. Review: Diagnostic accuracy of PCR-based detection tests for Helicobacter Pylori in stool samples. *Helicobacter* 2017; **22** [PMID: 28961384 DOI: 10.1111/hel.12444]

14 **Vécsei A**, Innerhofer A, Binder C, Gizci H, Hammer K, Bruckdorfer A, Riedl S, Gadner H, Hirschl AM, Makristathis A. Stool polymerase chain reaction for Helicobacter pylori detection and clarithromycin susceptibility testing in children. *Clin Gastroenterol Hepatol* 2010; **8**: 309-312 [PMID: 20005978 DOI: 10.1016/j.cgh.2009.12.002]

15 **Lottspeich C**, Schwarzer A, Panthel K, Koletzko S, Rüssmann H. Evaluation of the novel Helicobacter pylori ClariRes real-time PCR assay for detection and clarithromycin susceptibility testing of H. pylori in stool specimens from symptomatic children. *J Clin Microbiol* 2007; **45**: 1718-1722 [PMID: 17392440 DOI: 10.1128/JCM.00103-07]

16 **Noguchi N**, Rimbara E, Kato A, Tanaka A, Tokunaga K, Kawai T, Takahashi S, Sasatsu M. Detection of mixed clarithromycin-resistant and -susceptible Helicobacter pylori using nested PCR and direct sequencing of DNA extracted from faeces. *J Med Microbiol* 2007; **56**: 1174-1180 [PMID: 17761479 DOI: 10.1099/jmm.0.47302-0]

17 **Booka M**, Okuda M, Shin K, Miyashiro E, Hayashi H, Yamauchi K, Tamura Y, Yoshikawa N. Polymerase chain reaction--restriction fragment length polymorphism analysis of clarithromycin-resistant Helicobacter pylori infection in children using stool sample. *Helicobacter* 2005; **10**: 205-213 [PMID: 15904478 DOI: 10.1111/j.1523-5378.2005.00312.x]

18 **Rimbara E**, Noguchi N, Yamaguchi T, Narui K, Kawai T, Sasatsu M. Development of a highly sensitive method for detection of clarithromycin-resistant Helicobacter pylori from human feces. *Curr Microbiol* 2005; **51**: 1-5 [PMID: 15971095 DOI: 10.1007/s00284-004-4488-z]

19 **Scaletsky IC**, Aranda KR, Garcia GT, Gonçalves ME, Cardoso SR, Iriya K, Silva NP. Application of real-time PCR stool assay for Helicobacter pylori detection and clarithromycin susceptibility testing in Brazilian children. *Helicobacter* 2011; **16**: 311-315 [PMID: 21762271 DOI: 10.1111/j.1523-5378.2011.00845.x]

20 **Giorgio F**, Ierardi E, Sorrentino C, Principi M, Barone M, Losurdo G, Iannone A, Giangaspero A, Monno R, Di Leo A. Helicobacter pylori DNA isolation in the stool: an essential pre-requisite for bacterial noninvasive molecular analysis. *Scand J Gastroenterol* 2016; **51**: 1429-1432 [PMID: 27687850 DOI: 10.1080/00365521.2016.1216592]

21 **Bossuyt PM**, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig L, Lijmer JG, Moher D, Rennie D, de Vet HC, Kressel HY, Rifai N, Golub RM, Altman DG, Hooft L, Korevaar DA, Cohen

JF; STARD Group. STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. *BMJ* 2015; **351**: h5527 [PMID: 26511519 DOI: 10.1136/bmj.h5527]

22   **Stanghellini V**, Chan FK, Hasler WL, Malagelada JR, Suzuki H, Tack J, Talley NJ. Gastroduodenal Disorders. *Gastroenterology* 2016; **150**: 1380-1392 [PMID: 27147122 DOI: 10.1053/j.gastro.2016.02.011]

23   **Gatta L**, Vakil N, Ricci C, Osborn JF, Tampieri A, Perna F, Miglioli M, Vaira D. Effect of proton pump inhibitors and antacid therapy on 13C urea breath tests and stool test for Helicobacter pylori infection. *Am J Gastroenterol* 2004; **99**: 823-829 [PMID: 15128344 DOI: 10.1111/j.1572-0241.2004.30162.x]

24   **Graham DY**, Opekun AR, Hammoud F, Yamaoka Y, Reddy R, Osato MS, El-Zimaity HM. Studies regarding the mechanism of false negative urea breath tests with proton pump inhibitors. *Am J Gastroenterol* 2003; **98**: 1005-1009 [PMID: 12809820 DOI: 10.1111/j.1572-0241.2003.07424.x]

25   **Malfertheiner P**. Diagnostic methods for H. pylori infection: Choices, opportunities and pitfalls. *United European Gastroenterol J* 2015; **3**: 429-431 [PMID: 26535120 DOI: 10.1177/2050640615600968]

26   **Gisbert JP**, Pajares JM. Review article: 13C-urea breath test in the diagnosis of Helicobacter pylori infection -- a critical review. *Aliment Pharmacol Ther* 2004; **20**: 1001-1017 [PMID: 15569102 DOI: 10.1111/j.1365-2036.2004.02203.x]

27   **Li ZX**, Huang LL, Liu C, Formichella L, Zhang Y, Wang YM, Zhang L, Ma JL, Liu WD, Ulm K, Wang JX, Zhang L, Bajbouj M, Li M, Vieth M, Quante M, Zhou T, Wang LH, Suchanek S, Soutschek E, Schmid R, Classen M, You WC, Gerhard M, Pan KF. Cut-off optimization for 13 C-urea breath test in a community-based trial by mathematic, histology and serology approach. *Sci Rep* 2017; **7**: 2072 [PMID: 28522798 DOI: 10.1038/s41598-017-02180-7]

28   **Cohen JF**, Korevaar DA, Altman DG, Bruns DE, Gatsonis CA, Hooft L, Irwig L, Levine D, Reitsma JB, de Vet HC, Bossuyt PM. STARD 2015 guidelines for reporting diagnostic accuracy studies: explanation and elaboration. *BMJ Open* 2016; **6**: e012799 [PMID: 28137831 DOI: 10.1136/bmjopen-2016-012799]

29   **Buderer NM**. Statistical methodology: I. Incorporating the prevalence of disease into the sample size calculation for sensitivity and specificity. *Acad Emerg Med* 1996; **3**: 895-900 [PMID: 8870764]

30   **Beckman E**, Saracino I, Fiorini G, Clark C, Slepnev V, Patel D, Gomez C, Ponaka R, Elagin V, Vaira D. A Novel Stool PCR Test for Helicobacter pylori May Predict Clarithromycin Resistance and Eradication of Infection at a High Rate. *J Clin Microbiol* 2017; **55**: 2400-2405 [PMID: 28515219 DOI: 10.1128/JCM.00506-17]

31   **Ierardi E**, Giorgio F, Losurdo G, Di Leo A, Principi M. How antibiotic resistances could change Helicobacter pylori treatment: A matter of geography? *World J Gastroenterol* 2013; **19**: 8168-8180 [PMID: 24363506 DOI: 10.3748/wjg.v19.i45.8168]

32   **Brennan DE**, Omorogbe J, Hussey M, Tighe D, Holleran G, O'Morain C, Smith SM, McNamara D. Molecular detection of Helicobacter pylori antibiotic resistance in stool vs biopsy samples. *World J Gastroenterol* 2016; **22**: 9214-9221 [PMID: 27895408 DOI: 10.3748/wjg.v22.i41.9214]

ORIGINAL ARTICLE

Observational Study

Long-term outcomes of endoscopic resection for small (≤ 4.0 cm) gastric gastrointestinal stromal tumors originating from the muscularis propria layer

Yu Zhang, Xin-Li Mao, Xian-Bin Zhou, Hai Yang, Lin-Hong Zhu; Guang Chen, Li-Ping Ye

Yu Zhang, Xin-Li Mao, Xian-Bin Zhou, Li-Ping Ye, Department of Gastroenterology, Taizhou Hospital of Zhejiang Province, Wenzhou Medical University, Linhai 317000, Zhejiang Province, China

Hai Yang, Department of Radiology, Enze Hospital, Wenzhou Medical University, Taizhou city 318000, Zhejiang Province, China

Lin-Hong Zhu, Department of Medical Administration, Taizhou Hospital of Zhejiang Province, Wenzhou Medical University, Linhai 317000, Zhejiang Province, China

Guang Chen, Department of Surgery, Taizhou Hospital of Zhejiang Province, Wenzhou Medical University, Linhai 317000, Zhejiang Province, China

ORCID number: Yu Zhang (0000-0002-5522-3178); Xin-Li Mao (0000-0001-8341-2198); Xian-Bin Zhou (0000-0001-8674-0592); Hai Yang (0000-0003-3485-3872); Lin-Hong Zhu (0000-0002-3447-6707); Guang Chen (0000-0002-2605-5953); Li-Ping Ye (0000-0002-7938-0960).

Author contributions: Ye LP performed and analyzed the endoscopy operation; Zhang Y designed the study and drafted the manuscript; Mao XL performed postoperative management; Zhou XB and Zhu LH enrolled patients and acquired the follow-up data; Yang H and Chen G acquired the follow-up data.

Institutional review board statement: The study was reviewed and approved by the Institutional Medical Ethics Review Board of Taizhou Hospital of Zhejiang Province.

Informed consent statement: All participants provided informed consent prior to study enrollment.

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: No additional data are available.

STROBE statement: The STROBE Statement have been adopted.

Correspondence to: Li-Ping Ye, MBBS, Chief Doctor, Professor, Department of Gastroenterology, Taizhou Hospital of Zhejiang Province, Wenzhou Medical University, 150 Ximen Street, Linhai 317000, Zhejiang Province, China. xmcx783@126.com
Telephone: +86-13566866269
Fax: +86-576-85199171

Abstract

AIM
To evaluate the long-term efficacy of endoscopic resection (ER) for small (≤ 4.0 cm) gastric gastrointestinal stromal tumors (GISTs) originating from the muscularis propria layer.

METHODS
Between June 2005 and February 2015, we retrospectively analyzed 229 consecutive patients with gastric MP-GISTs who underwent ER with a follow-up at least 36 mo. The

main outcome measurements included complete resection rate, complications, and long-term follow-up outcomes.

RESULTS

ER included endoscopic muscularis excavation in 179 cases, endoscopic full-thickness resection in 32 cases, and submucosal tunneling endoscopic resection in 18 cases. The median size of GISTs was 1.90 cm. Of the 229 GISTs, 147 were very low risk, 72 were low risk, 8 were intermediate risk, and 2 were high risk. Short-term outcomes showed the complete resection rate was 96.5%, and 8 patients (3.5%) had complications. Of the 8 patients with complications, only one patient required surgical intervention. Long-term outcomes showed 225 patients were actively followed-up until composition of this manuscript. The remaining 4 patients were lost because of unrelated death. During the follow-up period (median, 57 mo), no residual, recurrent lesions, or distant metastasis were detected in any patients. Binary logistic regression analysis showed tumor size was a risk factor associated with a high mitotic index (≥ 5/50 HPF) of GISTs (P = 0.002).

CONCLUSION

ER seems to be an effective and safe method for gastric MP-GISTs ≤ 4.0 cm, and, for some intermediate or high risk GISTs, adjuvant therapy and/or additional surgery might be required to reduce the risk of recurrence or metastasis.

Core tip: The long-term effectiveness of endoscopic resection for small (≤ 4.0 cm) gastric gastrointestinal stromal tumors originating from the muscularis propria layer (MP-GISTs) still remains debatable. In our study, we included a larger sample size with a longer follow-up period to assess the long-term safety and efficacy of ER for gastric MP-GIST. Long-term outcomes showed 225 patients were actively followed-up until composition of this manuscript. The remaining 4 patients were lost because of unrelated death. During the follow-up period (median, 57 mo), no residual, recurrent lesions, or distant metastasis were detected in any patients. Endoscopic resection seems to be an effective and safe method for gastric MP-GISTs ≤ 4.0 cm, and, for some intermediate or high risk GISTs, adjuvant therapy and/or additional surgery might be required to reduce the risk of recurrence or metastasis.

INTRODUCTION

Recently, endoscopic resection (ER) has become one of the prevailing treatments for small (≤ 4.0 cm) gastric gastrointestinal stromal tumors originating from the muscularis propria layer (MP-GISTs), in many countries[1-6]. However, its long-term effectiveness still remains debatable. Some surgeons have posited that ER can result in insufficient margins that would likely increase the risk of tumor recurrence or implantation metastasis. Thus, laparoscopic wedge resection (LWR) is still recommended for treating small gastric MP-GISTs[7-9]. Nevertheless, LWR is associated with a number of serious long-term complications. Especially when the tumor is located near or in the gastric cardia or pylorus, resection of the gastric cardia or pylorus might lead to irreparable damage to the cardioesophageal sphincter or pylori sphincter, leaving patients prone to certain diseases associated with digestive fluid reflux[7, 9,10].

Although recurrent tumors or implantation metastasis events were extremely rare according to the available literature where ER has been used for small gastric MP-GISTs, such study series were relatively small or only had a short follow-up period of less than 36 mo[2,11-14]. Since June 2005, our endoscopy center has been using ER for small (≤ 4.0 cm) upper gastrointestinal subepithelial tumors originating from the muscularis propria layer (MP-SETs). Up to December 2017, we had completed 1021 cases of ER for small (≤ 4.0 cm) upper gastrointestinal MP-SETs. Within those 1021 cases, we selected 229 consecutive patients who had gastric MP-GISTs less than 4.0 cm with at least 36 mo of follow-up after ER, and demonstrated the long-term safety and efficacy of ER for this type of tumor.

MATERIALS AND METHODS

Study design and patients

This retrospective cohort study was approved by the Ethics Committee of Taizhou Hospital, Wenzhou Medical College. Data from patients with gastric MP-GISTs who underwent ER were reviewed between June 2005 and February 2015 at the Information Systems Department of Taizhou Hospital of Zhejiang Province. Patients were included if they met all of the following criteria: (1) The patient had a gastric MP-GIST that was diagnosed histopathologically as a GIST after ER; (2) the tumors were 1.0-4.0 cm in size and did not exhibit bleeding, ulceration, or scarring; and (3) the patient had no evidence of lymph node metastasis assessed by endoscopic ultrasonography (EUS) or computed tomography (CT) examination preoperatively and also did not have other malignant tumors. According to

the inclusion criteria, 229 consecutive patients with gastric MP-GISTs were included. Before the endoscopic procedure, informed consent was obtained from all patients in accordance with the institutional protocol.

Endoscopic procedures

ER included endoscopic muscularis excavation (EME), endoscopic full-thickness resection (EFTR), and submucosal tunneling endoscopic resection (STER). Between June 2005 and July 2011, patients with gastric MP-GISTs usually were treated by EME. If the tumor had the characteristic of extensive connection to the underlying MP or serosal layer, EFTR was performed to resect it completely. Since August 2011, if the tumor was located in the cardia, the proximal body, or fundus of the stomach near the cardia where a submucosal tunnel can be established, we applied the STER technique to resect it[1].

All ER procedures were performed under general anesthesia by a highly skilled endoscopist (LPY) in the operating room. The basic equipment and accessories included a single-channel endoscope (Q-260 J, Olympus) and/or a dual-channel endoscope (GIF-2T240, Olympus), an electrosurgical generator (ICC 200; ERBE, Tübingen, Germany), argon plasma coagulation (APC 300, ERBE), and a carbon dioxide insufflator (Olympus Optical).

When performing EME, injection solution (100 mL saline plus 2 mL indigo carmine and 1 mL epinephrine) was used to make a submucosal elevation after placing marking several dots around the tumor. A cross or circumferential mucosal incision was made to reveal the lesion with a hook knife (KD-620LR; Olympus), and then a circumferential resection was performed along the edge of the lesion until the lesion was completely excavated from the muscularis propria (MP) layer with an insulated-tip knife (KD-611L, IT2; Olympus) or a hybrid knife (ERBE)[15].

When performing EFTR (Figure 1), several dots and a submucosal elevation were made similar to the EME procedure, and then a circumferential incision was made along the marking dots until the intraluminal side of the lesion was fully revealed. Subsequently, a small puncture was first made in the seromuscular layer of the lesion with an insulated-tip knife (KD-611L, IT2; Olympus), and then the resection was continuously performed along the puncture. A snare resection was made to completely remove the lesion after three-quarters of the circumference of the tumor was resected. Finally, the gastric wall defect was closed with several clips and an endoloop or an over-the-scope clip (OTSC) after tumor removal[15].

When performing STER (Figure 2), the lesion was first marked by submucosal injection of methylene blue, and then a 2 cm longitudinal mucosal incision was made 5 cm above the lesion with a hook knife (KD-620LR, Olympus), which was used as the entry point. Subsequently, a submucosal tunnel was created between the submucosal and muscular layers to the lesion using

a hybrid knife, and the lesion was excavated from the MP layer through the submucosal tunnel until complete lesion separation. Lastly, the mucosal incision site was closed with clips after adequate hemostasis and tumor removal[15].

Outcome measurements

The main measurements of this study were divided into two parts: short-term outcomes and long-term outcomes. Short-term outcomes included complete resection and complications. Complete resection refers to tumors removed en bloc with no apparent residual tumor at the resection site and with negative margins upon pathologic examination[1]. In this study, complications were identified as perioperative bleeding, delayed bleeding, localized peritonitis, or perforation requiring surgical intervention. Perforation that did not require surgical intervention and could be closed by endoscopic methods was not considered a complication[2]. Long-term outcomes included rates of local recurrence and distant metastasis in patients who were followed up for more than 3 years. Local recurrence was defined as the identification of a lesion located on or adjacent to the scar of the previous ER, which was subsequently confirmed *via* biospy[1].

Follow-up

All 229 patients were included in a prospectively maintained database at the Medical Information Centre of Taizhou Hospital of Zhejiang Province. Every patient with a gastric MP-GIST was examined with gastroscopy to check for wound healing at 3 mo and 6 mo after ER, and every patient also underwent EUS to check for residual lesions at 3 mo. Subsequently, gastroscopy and/or EUS was performed to look for local recurrent lesions, and abdominal ultrascan and/or CT were used to check distant metastasis every year, indefinitely.

Statistical analysis

Data were analyzed using SPSS 20.0 software (SPSS Inc., Chicago, IL, United States). For descriptive statistics, the mean was used in the case of a normal distribution of variables, whereas the median (interquartile range) was used for variables with a skewed distribution. Binary logistic regression analysis was used to test for effect associations among independent variables and a high mitotic index (5/50 HPF) of GISTs. A *P* value < 0.05 was considered statistically significant.

RESULTS

Patient and tumor characteristics

A total of 229 consecutive patients with gastric MP-GISTs were included in this study (143 females, 86 males; mean age 54.9 ± 10.8 years old). As displayed in Table 1, 29 GISTs were located in the gastric cardia, 118 in the gastric fundus, 72 in the gastric body, and 10 in the antrum. The median size of GIST specimens was 1.90 cm (interquartile range 1.55-2.40 cm). All 229 GISTs had

Table 1 Demographic and clinicopathologic features of 229 gastric MP-GISTs *n* (%)

Parameters	Endoscopic resection (*n* = 229)
Age (yr), mean ± SD	54.9 ± 10.8
Gender	
Male	86
Female	143
Tumor size (cm), median (interquartile range)	1.90 (1.55-2.40)
Tumor location	
Cardia	29 (12.7)
Fundus	118 (51.5)
Body	72 (32.1)
Antrum	10 (3.3)
Tumor growth pattern	
Intraluminal growth	178 (77.7)
Extraluminal growth	51 (22.3)
EUS characteristics	
Homogeneous echo	197 (86.0)
Inhomogeneous echo	32 (14.0)
Mitotic index	
< 5/50 HPF	219 (95.6)
5-10/50 HPF	8 (3.5)
≥ 10/50 HPF	2 (0.9)
NIH risk classification	
Very low	147 (64.2)
Low risk	72 (31.4)
Intermediate risk	8 (3.5)
High risk	2 (0.9)

EUS: Endoscopic ultrasonography.

a low-level echo, including a homogeneous echo in 197 cases and an inhomogeneous echo in 32 cases.

219 out of 229 GISTs had a mitotic count of < 5 per 50 high-power fields, 8 out of 229 GISTs had a count of 5-10 per 50 high-power fields, and 2 out of 229 GISTs had a count of ≥ 10 per 50 high-power fields. According to the NCCN guidelines[16], 147 GISTs were very low risk, 72 were low risk, 8 were intermediate risk, and 2 were high risk.

Treatment outcomes

In this study, 179 GISTs were treated with EME, 32 GISTs were treated with EFTR, and 18 GISTs were treated with STER. The mean time of ER procedure was 52.8 ± 16.1 min. Complete resection by ER was achieved in 221 lesions (96.5%). Among the other 8 GISTs without complete resection, 3 GISTs were resected in one piece during EME technique, but the tumor margin could not be evaluated definitively because of electrocautery, and the other 5 GISTs were resected piecemeal during the EME procedure. Of the 8 GISTs, 5 were located in the gastric fundus and 3 in the gastric body. The size of these GISTs in diameter was ranged 2.7 cm to 3.6 cm. According to the NCCN guidelines, these 8 GISTs were all low risk. Because those patients were unwilling to accept the potential risk of the surgery, further surgical resection was not performed after pathological examination. The median time from endoscopic treatment to a no-residue diet

was 4 d (range 1-10 d, interquartile range 3-6 d). The median length of hospital stay after ER was 5 d (range 1-14 d, interquartile range 3-7 d).

Complications

In this study, 8 patients had complications (3.5%, 8/229), including 5 patients with perioperative bleeding (2.2%, 5/229), 2 patients with localized peritonitis (0.9%, 2/229), and one patient with delayed bleeding (0.4%, 1/229). Of the 8 patients with complications, only one patient with perioperative bleeding was converted to laparoscopic surgery (0.4%), and the other 7 patients were managed successfully by endoscopic methods and conservative treatment (gastrointestinal decompression, the intravenous infusion of antibiotics and esomeprazole). No patients had other serious complications.

Long-term outcomes

In this study, 225 out of 229 patients with gastric MP-GISTs were actively followed-up until composition of this manuscript. The remaining 4 patients were lost to follow-up because of unrelated death. Two patients with very low risk GISTs died of cordis and cerebral accident at 53 and 86 mo post-endoscopic procedure, respectively. Another patient with a low risk GIST died of pneumonia at 47 mo, and the last patient with a very low risk GIST died from traffic-accident at 59 mo.

The median follow-up period after ER was 57 mo (range 36-132 mo; interquartile range 46-71 mo. In this study, 2 patients with high-risk GISTs took imatinib mesylate to prevent recurrence or metastasis, whereas the other 8 patients with intermediate-risk GISTs were unable to take imatinib mesylate because they were unable afford the medication. During the follow-up period, no residual, recurrent lesions, or distant metastasis were detected in any patients, including 8 patients without complete resection. Furthermore, all 229 patients who underwent ER maintained their previous quality of life.

Risk factors associated with a high mitotic index (≥ 5/50 HPF) of GISTs

Risk factors analyzed included: Age (≤ 40 years old, 41-60 years old, 60 years old), gender (male, female), tumor size (< 2.0, 2.0-3.0, and ≥ 3.0 cm), tumor location (antrum with an OR value defined as 1, compared with body, fundus, and cardia), and tumor growth pattern (intraluminal, extraluminal). Among the analyzed factors, tumor size was the only risk factor associated with a high mitotic index (≥ 5/50 HPF) of GISTs (OR = 6.675; 95%CI: 2.047-21.771; *P* = 0.002; Table 2).

DISCUSSION

Gastric MP-GISTs are among the most common gastric MP-SETs, which account for about 13.0%-71.4% of all gastric SETs[1,5,10,17,18]. A proportion of gastric MP-
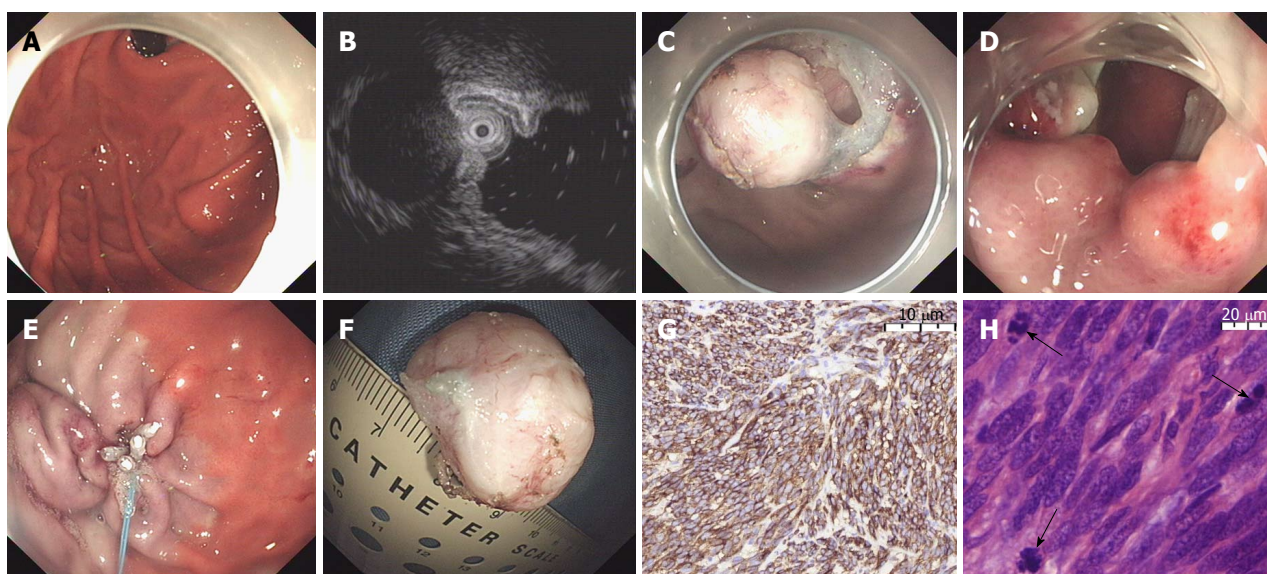
Figure 1 Endoscopic full-thickness resection for a gastrointestinal stromal tumors located in the gastric fundus. A: Endoscopy showed a SET was located in the gastric fundus; B: EUS showed the same tumor mainly bulged into the extraluminal space and had an extensive connection to the MP layer of the stomach; C: The tumor, including its underlying MP and serosa, was resected; D and E: The gastric wall defect was closed using clips combined with an endoloop; F: The resection specimen was a 2.7 cm tumor; G: Immunohistochemistry showed that CD117 was present in most tumor cells (original magnification × 100); H: Mitotic figures could be found easily (original magnification × 400). EUS: Endoscopic ultrasonography.
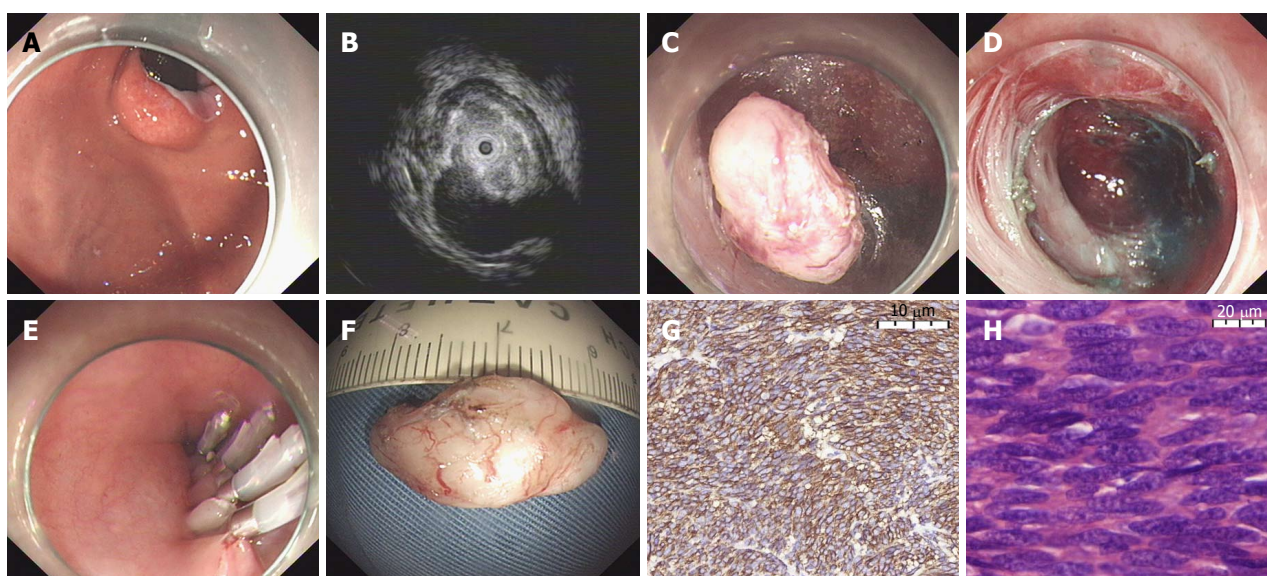

Figure 2 Submucosal tunneling endoscopic resection for a gastrointestinal stromal tumors located in the gastric cardia. A: Endoscopy showed a SET was located in the gastric cardia; B: EUS showed the same tumor mainly bulged into the extraluminal space and had an extensive connection to the MP layer of the stomach; C: The tumor was resected from the MP layer *via* submucosal tunneling; D and E: The mucosal incision site was closed with several clips after tumor removal; F: The resection specimen was a 2.4 cm tumor; G: Immunohistochemistry showed that CD34 was present in most tumor cells (original magnification × 100); H: Mitotic figures could not be found (original magnification × 400). EUS: Endoscopic ultrasonography.

GISTs are known to possess some degree of malignant potential, which usually cannot be diagnosed accurately before surgical or endoscopic removal. Resection is often recommended because it not only provides an accurate diagnosis, but also may be curative if the lesion is removed completely[10,15]. Currently, ER is being increasingly used for gastric MP-GIST removal. However, additional evidence is required to support the long-term effectiveness of ER for the treatment of gastric GISTs. Compared with the published studies, our study included a larger sample size with a longer follow-up period to assess the long-term safety and efficacy of ER for gastric MP-GIST, which would increase the evidence and support for the use of ER to treat/remove gastric MP-GISTs.

Short-term outcomes of this study showed complete resection by ER was achieved in 221 lesions (96.5%), and complications occurred in 8 patients (3.5%), including 5

Table 2 Risk factors associated with a high mitotic index (5/50 HPF) of gastrointestinal stromal tumors

	High mitotic index (> 5/50 HPF)		
	Odds ratio	95%CI	*P* value
Age, yr (≤ 40, 40 to ≤ 60, and > 60)	-	-	0.756
Gender (male, female)	-	-	0.982
Tumor size, cm (< 2.0, 2.0-3.0, and ≥ 3.0)	6.675	2.047-21.771	0.002
Tumor location (cardia, fundus, body, and antrum)	-	-	0.505
Tumor growth pattern (intraluminal, extraluminal)	-	-	0.069

Table 3 Detailed information of studies evaluating endoscopic resection for gastric gastrointestinal stromal tumors

Author	Publication year	Study design	Case *n*	Endoscopic procedure	Tumor size, mean ± SD (cm)	Pathologic risk grade, *n*	Complete resection *n* (%)	surgical intervention *n* (%)	Recurrence *n* (%)	Follow-up (mo)
Feng *et al*[14]	2015	RS	50	Endoscopic resection	< 2	MI ≤ 5, 41 MI > 5, 9	NA	1 (2.0)	0	32[1] (12-65)
Shen *et al*[13]	2015	RS	32	ER	1.70 ± 0.36	Very low, 9 low, 18 intermediate, 3 high, 2	32 (100)	1 (3.1)	1 (3.1)	31.5[1] (2-53)
Joo *et al*[4]	2016	RS	90	ESD, 72 STER, 8 EMR, 7 EFTR, 2 Polypectomy, 1	2.3 ± 1.2	Very low, 45 Low, 28 Intermediate, 1 High, 6	88 (97.8)	5 (5.6)	2 (2.2)	46.0 ± 28.5
Tan *et al*[21]	2017	RS	52	STER, 20 EFTR, 32	STER, 17.8 ± 7.2; EFTR, 15.4 ± 6.6	Low, 26 Intermediate, 26	En bloc 50 (96.2)	1 (1.9)	1 (1.9)	10.9 ± 7.8 23.8 ± 18.6
An *et al*[11]	2017	RS	168	ESD	1.5[1] (0.5-6)	Very low, 117 Low, 37 Intermediate, 14	En bloc 168 (100)	0	0	25[1] (6-67)
Balde *et al*[18]	2017	RS	30	ESD	1.5[1] (1.0-1.8)	Very low, 22 Low, 4 Intermediate, 4	27 (90.0)	NA	2 (6.7)	57.9 (± 28.9)
Meng *et al*[19]	2017	RS	75	ESD	1.44 ± 0.67	NA	NA	NA	2 (2.7)	3.3 yr[1] 1-7 yr
Andalib *et al*[2]	2018	RS	12	EN, 5 EFTR, 7	2.4[1] (1.0 - 5.0)	Low, 11 Intermediate, 1	11 (91.7)	0	0	12[1] (6.5-24)
This study	-	RS	229	EME, 179 EFTR, 32 STER, 18	1.90[1] (1.0-4.0)	Very low, 147 Low, 72 Intermediate, 8 High, 2	221 (96.5)	1	0	57[1] (46-71)

[1]Median. RS: Retrospective; ER: Endoscopic resection; ESD: Endoscopic submucosal dissection; STER: Submucosal tunneling endoscopic resection; EMR: Endoscopic mucosal resection; EN: Endoscopic enucleation; EME: Endoscopic muscularis excavation; EFTR: Endoscopic full-thickness resection; MI: Mitotic index.

patients with perioperative bleeding (2.2%), 2 patients with localized peritonitis (0.9%), and one patient with delayed bleeding (0.4%, 1/229). Among the 8 patients with complications, only one patient with perioperative bleeding required a conversion to laparoscopic surgery. No patients had other serious complications. These short-term outcomes were consistent with previously published findings from a number studies evaluating the use of ER for the treatment of gastric GISTs[2,4,11,12,14]. According to these short-term outcomes, ER is a feasible treatment for gastric MP-GISTs.

The risk of local recurrence associated with ER for gastric GISTs was a major concern of some surgeons. Recently, a number of studies using ER to treat gastric GISTs have shown that the recurrence rate ranged from 0 to 6.7% (Table 3)[2-4,11-14,19]. In 3 of these studies with a mean/median follow-up period of ≥ 36 mo, the recurrence rate ranged between 2.2% and 6.7%[3,4,19]. In our study, no patient experienced a local recurrence or distant metastasis during a median follow-up period

of 57 mo. Some differences in the rate of recurrence might be explained by the single-center retrospective design, different inclusion criteria applied in different studies, different pathologic risk grades of gastric GISTs, and different ER methods performed in different endoscopic centers.

Complete resection might be a key factor associated with a low recurrence rate in patients with gastric GISTs. Our experience of complete resection for gastric MP-GISTs can be concluded as follows. First, the tumor size of gastric MP-GISTs might be no more than 4.0 cm in diameter. When the tumor size is > 4 cm in diameter, it is very difficult to remove the tumor en bloc with an endoscopic approach, because of the limitations of the cardia and esophagus space[1,2]. Meanwhile, larger tumor size is associated with certain disadvantages, such as a narrower endoscopic view, higher complication rate, and longer endoscopic resection time. Therefore, in our endoscopy center, surgical resection still is the first choice for patients with

gastric GISTs > 4 cm. Second, the tumor capsule must be kept intact during ER. Submucosal injection solution was repeatedly injected into the surrounding tissue, which allowed for the differentiation of the MP layer from the tumor mass, which avoided tumor capsule rupture when making a circular resection around the lesion. Third, the tumor should be assessed by EUS and CT before ER. For some lesions with high risk features (irregular border, cystic spaces, echogenic foci, and internal heterogeneity) identified on EUS or metastasis confirmed by CT, ER is absolutely contraindicated. Finally, EFTR is recommended when the gastric MP-GIST has extraluminal growth or is tightly connected to the underlying MP or serosal layer.

According to several previous studies, the mitotic index is an important prognostic factor for GISTs[4,13,20]. Several previous studies reported that even small GISTs (< 2.0 cm) have malignant potential with a high mitotic index[4,13]. In this study, we found 8 out of 229 GISTs had an index of 5-10 per 50 high-power fields, and 2 out of 229 GISTs had an index of ≥ 10 per 50 high-power fields. Subsequently, we analyzed the risk factors associated with the high mitotic index (≥ 5/50 HPF) and found that tumor size was significantly and independently related to a high mitotic index (OR = 6.675, *P* = 0.002). Thus, tumor size is still another important factor associated with the malignant potential of small gastric GISTs, which might affect the long term prognosis of patients with gastric GISTs. For some GISTS, classified as intermediate or high risk, adjuvant therapy (imatinib mesylate, *etc.*) and/or additional surgery are recommended to reduce the risk of recurrence after ER[21,22].

However, this study had a few limitations. First, this is a single-center, retrospective cohort study, and a selection bias may be present, even though the data were collected from a prospectively maintained database. Second, the lack of randomization might be another factor contributing to selection bias. Third, 4 out of 229 patients discontinued follow-up because of unrelated death. Finally, our institution is a tertiary endoscopic center in Zhejiang Province, and all endoscopic operations were performed by an experienced endoscopist. Thus, the results in this study might not apply to all centers. Therefore, a randomized, controlled, multicenter study is needed to evaluate the safety of ER for small (≤ 4.0 cm) gastric GISTs.

In conclusion, ER might be an effective and safe therapeutic method for patients with gastric MP-GISTs ≤ 4.0 cm, and for some patients with intermediate or high risk GISTs, adjuvant therapy (imatinib mesylate, *etc.*) and/or additional surgery might be required to reduce the risk of recurrence.

ARTICLE HIGHLIGHTS

Research background
The long-term effectiveness of endoscopic resection for small gastric gastrointestinal stromal tumors originating from the muscularis propria layer (MP-GISTs) still remains debatable.

Research motivation
A larger sample size with a longer follow-up period were included to assess the long-term safety and efficacy of ER for gastric MP-GIST

Research objectives
Evaluate the long-term efficacy of ER for small gastric gastrointestinal stromal tumors originating from the muscularis propria layer.

Research methods
We retrospectively analyzed 229 consecutive patients with gastric MP-GISTs who underwent ER with a follow-up at least 36 mo, between June 2005 and February 2015. The main outcome measurements included complete resection rate, complications, and long-term follow-up outcomes.

Research results
The outcomes showed that 225 patients were actively followed-up. The remaining 4 patients were lost because of unrelated death. During the follow-up period, no residual, recurrent lesions, or distant metastasis were detected in any patients.

Research conclusions
Endoscopic resection seems to be an effective and safe method for gastric MP-GISTs ≤ 4.0 cm, and for some intermediate or high risk GISTs, adjuvant therapy might be required to reduce the risk of recurrence or metastasis.

Research perspectives
Endoscopic resection might be an effective and safe therapeutic method for patients with gastric MP-GISTs ≤ 4.0 cm, and for some patients with intermediate or high risk GISTs, adjuvant therapy (imatinib mesylate, *etc.*) and/or additional surgery might be required to reduce the risk of recurrence.

REFERENCES

1   **Ye LP**, Zhang Y, Luo DH, Mao XL, Zheng HH, Zhou XB, Zhu LH. Safety of Endoscopic Resection for Upper Gastrointestinal Subepithelial Tumors Originating from the Muscularis Propria Layer: An Analysis of 733 Tumors. *Am J Gastroenterol* 2016; **111**: 788-796 [PMID: 26782819 DOI: 10.1038/ajg.2015.426]

2   **Andalib I**, Yeoun D, Reddy R, Xie S, Iqbal S. Endoscopic resection of gastric gastrointestinal stromal tumors originating from the muscularis propria layer in North America: methods and feasibility data. *Surg Endosc* 2018; **32**: 1787-1792 [PMID: 28916847 DOI: 10.1007/s00464-017-5862-9]

3   **Balde AI**, Chen T, Hu Y, Redondo N JD, Liu H, Gong W, Yu J, Zhen L, Li G. Safety analysis of laparoscopic endoscopic cooperative surgery versus endoscopic submucosal dissection for selected gastric gastrointestinal stromal tumors: a propensity score-matched study. *Surg Endosc* 2017; **31**: 843-851 [PMID: 27492430 DOI: 10.1007/s00464-016-5042-3]

4   **Joo MK**, Park JJ, Kim H, Koh JS, Lee BJ, Chun HJ, Lee SW, Jang YJ, Mok YJ, Bak YT. Endoscopic versus surgical resection of GI stromal tumors in the upper GI tract. *Gastrointest Endosc* 2016; **83**: 318-326 [PMID: 26227928 DOI: 10.1016/j.gie.2015.07.034]

5   **Huang LY**, Cui J, Lin SJ, Zhang B, Wu CR. Endoscopic full-thickness resection for gastric submucosal tumors arising from the muscularis propria layer. *World J Gastroenterol* 2014; **20**: 13981-13986 [PMID: 25320536 DOI: 10.3748/wjg.v20.i38.13981]

6   **Schmidt A**, Bauder M, Riecken B, von Renteln D, Muehleisen H, Caca K. Endoscopic full-thickness resection of gastric subepithelial tumors: a single-center series. *Endoscopy* 2015; **47**: 154-158 [PMID: 25380509 DOI: 10.1055/s-0034-1390786]

7   **Kim DJ**, Lee JH, Kim W. Laparoscopic resection for 125 gastroduodenal submucosal tumors. *Ann Surg Treat Res* 2014; **86**: 199-205 [PMID: 24783179 DOI: 10.4174/astr.2014.86.4.199]

8   **Dressler JA**, Palazzo F, Berger AC, Stake S, Chaudhary A,

Chojnacki KA, Rosato EL, Pucci MJ. Long-term functional outcomes of laparoscopic resection for gastric gastrointestinal stromal tumors. *Surg Endosc* 2016; **30**: 1592-1598 [PMID: 26169640 DOI: 10.1007/s00464-015-4384-6]

9   **Cui JX**, Gao YH, Xi HQ, Cai AZ, Zhang KC, Li JY, Wei B, Chen L. Comparison between laparoscopic and open surgery for large gastrointestinal stromal tumors: A meta-analysis. *World J Gastrointest Oncol* 2018; **10**: 48-55 [PMID: 29375748 DOI: 10.4251/wjgo.v10.i1.48]

10   **Li QL**, Yao LQ, Zhou PH, Xu MD, Chen SY, Zhong YS, Zhang YQ, Chen WF, Ma LL, Qin WZ. Submucosal tumors of the esophagogastric junction originating from the muscularis propria layer: a large study of endoscopic submucosal dissection (with video). *Gastrointest Endosc* 2012; **75**: 1153-1158 [PMID: 22459663 DOI: 10.1016/j.gie.2012.01.037]

11   **An W**, Sun PB, Gao J, Jiang F, Liu F, Chen J, Wang D, Li ZS, Shi XG. Endoscopic submucosal dissection for gastric gastrointestinal stromal tumors: a retrospective cohort study. *Surg Endosc* 2017; **31**: 4522-4531 [PMID: 28374257 DOI: 10.1007/s00464-017-5511-3]

12   **Tan Y**, Tang X, Guo T, Peng D, Tang Y, Duan T, Wang X, Lv L, Huo J, Liu D. Comparison between submucosal tunneling endoscopic resection and endoscopic full-thickness resection for gastric stromal tumors originating from the muscularis propria layer. *Surg Endosc* 2017; **31**: 3376-3382 [PMID: 27864722 DOI: 10.1007/s00464-016-5350-7]

13   **Shen C**, Chen H, Yin Y, Chen J, Han L, Zhang B, Chen Z, Chen J. Endoscopic versus open resection for small gastric gastrointestinal stromal tumors: safety and outcomes. *Medicine* (Baltimore) 2015; **94**: e376 [PMID: 25569663 DOI: 10.1097/md.0000000000000376]

14   **Feng F**, Liu Z, Zhang X, Guo M, Xu G, Ren G, Hong L, Sun L, Yang J, Zhang H. Comparison of Endoscopic and Open Resection for Small Gastric Gastrointestinal Stromal Tumor. *Transl Oncol* 2015; **8**: 504-508 [PMID: 26692532 DOI: 10.1016/j.tranon.2015.11.008]

15   **Zhang Y**, Ye LP, Mao XL. Endoscopic treatments for small gastric subepithelial tumors originating from muscularis propria layer. *World J Gastroenterol* 2015; **21**: 9503-9511 [PMID: 26327758 DOI: 10.3748/wjg.v21.i32.9503]

16   **Demetri GD**, von Mehren M, Antonescu CR, DeMatteo RP, Ganjoo KN, Maki RG, Pisters PW, Raut CP, Riedel RF, Schuetze S, Sundar HM, Trent JC, Wayne JD. NCCN Task Force report: update on the management of patients with gastrointestinal stromal tumors. *J Natl Compr Canc Netw* 2010; **8** Suppl 2: S1-41; quiz S42-4 [PMID: 20457867]

17   **He Z**, Sun C, Wang J, Zheng Z, Yu Q, Wang T, Chen X, Liu W, Wang B. Efficacy and safety of endoscopic submucosal dissection in treating gastric subepithelial tumors originating in the muscularis propria layer: a single-center study of 144 cases. *Scand J Gastroenterol* 2013; **48**: 1466-1473 [PMID: 24131359 DOI: 10.3109/00365521.2013.845796]

18   **Białek A**, Wiechowska-Kozłowska A, Pertkiewicz J, Polkowski M, Milkiewicz P, Karpińska K, Ławniczak M, Starzyńska T. Endoscopic submucosal dissection for treatment of gastric subepithelial tumors (with video). *Gastrointest Endosc* 2012; **75**: 276-286 [PMID: 22032850 DOI: 10.1016/j.gie.2011.08.029]

19   **Meng Y**, Li W, Han L, Zhang Q, Gong W, Cai J, Li A, Yan Q, Lai Q, Yu J, Bai L, Liu S, Li Y. Long-term outcomes of endoscopic submucosal dissection versus laparoscopic resection for gastric stromal tumors less than 2 cm. *J Gastroenterol Hepatol* 2017; **32**: 1693-1697 [PMID: 28220962 DOI: 10.1111/jgh.13768]

20   **Jeong IH**, Kim JH, Lee SR, Kim JH, Hwang JC, Shin SJ, Lee KM, Hur H, Han SU. Minimally invasive treatment of gastric gastrointestinal stromal tumors: laparoscopic and endoscopic approach. *Surg Laparosc Endosc Percutan Tech* 2012; **22**: 244-250 [PMID: 22678321 DOI: 10.1097/SLE.0b013e31825078f2]

21   **Tan Y**, Tan L, Lu J, Huo J, Liu D. Endoscopic resection of gastric gastrointestinal stromal tumors. *Transl Gastroenterol Hepatol* 2017; **2**: 115 [PMID: 29354772 DOI: 10.21037/tgh.2017.12.03]

22   **Zhang Q**, Gao LQ, Han ZL, Li XF, Wang LH, Liu SD. Effectiveness and safety of endoscopic resection for gastric GISTs: a systematic review. *Minim Invasive Ther Allied Technol* 2018; **27**: 127-137 [PMID: 28681655 DOI: 10.1080/13645706.2017.1347097]

SYSTEMATIC REVIEWS

Systematic overview of hepatitis C infection in the Middle East and North Africa

Karima Chaabna, Sohaila Cheema, Amit Abraham, Hekmat Alrouh, Albert B Lowenfels, Patrick Maisonneuve, Ravinder Mamtani

Karima Chaabna, Sohaila Cheema, Amit Abraham, Hekmat Alrouh, Ravinder Mamtani, Institute for Population Health, Weill Cornell Medicine-Qatar, Doha 24144, Qatar

Albert B Lowenfels, Department of Surgery and the Department of Family Medicine, New York Medical College, Valhalla, NY 10595, United States

Patrick Maisonneuve, Division of Epidemiology and Biostatistics, European Institute of Oncology, Milan 20141, Italy

ORCID number: Karima Chaabna (0000-0002-1443-207X); Sohaila Cheema (0000-0002-5874-4059); Amit Abraham (0000-0002-9696-8598); Hekmat Alrouh (0000-0003-2224 -149X); Albert B Lowenfels (0000-0003-3631-4530); Patrick Maisonneuve (0000-0002-5309-4704); Ravinder Mamtani (0000-0001-8507-281X).

Author contributions: Chaabna K, Cheema S and Mamtani R collectively contributed to the conception of the study; Chaabna K, Abraham A and Alrouh H were involved in the literature search, screening, and extraction steps; Analysis and manuscript drafting were implemented by Chaabna K with support from Cheema S, Lowenfels AB, Maisonneuve P, and Mamtani R; all authors read, edited, and approved the final manuscript.

Conflict-of-interest statement: The authors have declared that no competing interests exist.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Manuscript source: Invited manuscript

Correspondence to: Karima Chaabna, PhD, Institute for Population Health, Weill Cornell Medicine-Qatar, Qatar Foundation-Education City, Doha 24144, Qatar. kac2047@qatar-med.cornell.edu
Telephone: +974-44928309
Fax: +974-44928333

Abstract

AIM

To assess the quality of and to critically synthesize the available data on hepatitis C infections in the Middle East and North Africa (MENA) region to map evidence gaps.

METHODS

We conducted an overview of systematic reviews (SRs) following an a priori developed protocol (CRD42017076736). Our overview followed the preferred reporting items for systematic reviews and meta-analyses guidelines for reporting SRs and abstracts and did not receive any funding. Two independent reviewers systematically searched MEDLINE and conducted a multistage screening of the identified articles. Out of 5758 identified articles, 37 SRs of hepatitis C virus (HCV) infection in populations living in 20 countries in the MENA region published between 2008 and 2016 were included in our overview. The nine primary outcomes of interest were HCV antibody (anti-) prevalences and incidences in different at-risk populations; the HCV viremic (RNA positive) rate in HCV-positive individuals; HCV viremic prevalence in the general population (GP); the prevalence of HCV co-infection with the hepatitis B virus, human

immunodeficiency virus, or schistosomiasis; the HCV genotype/subtype distribution; and the risk factors for HCV transmission. The conflicts of interest declared by the authors of the SRs were also extracted. Good quality outcomes reported by the SRs were defined as having the population, outcome, study time and setting defined as recommended by the PICOTS framework and a sample size > 100.

RESULTS
We included SRs reporting HCV outcomes with different levels of quality and precision. A substantial proportion of them synthesized data from mixed populations at differing levels of risk for acquiring HCV or at different HCV infection stages (recent and prior HCV transmissions). They also synthesized the data over long periods of time (*e.g.*, two decades). Anti-HCV prevalence in the GP varied widely in the MENA region from 0.1% (study dates not reported) in the United Arab Emirates to 2.1%-13.5% (2003-2006) in Pakistan and 14.7% (2008) in Egypt. Data were not identified for Bahrain, Jordan, or Palestine. Good quality estimates of anti-HCV prevalence in the GP were reported for Algeria, Djibouti, Egypt, Iraq, Morocco, Pakistan, Syria, Sudan, Tunisia, and Yemen. Anti-HCV incidence estimates in the GP were reported only for Egypt (0.8-6.8 per 1000 person-year, 1997-2003). In Egypt, Morocco, and the United Arab Emirates, viremic rates in anti-HCV-positive individuals from the GP were approximately 70%. In the GP, the viremic prevalence varied from 0.7% (2011) in Saudi Arabia to 5.8% (2007-2008) in Pakistan and 10.0% (2008) in Egypt. Anti-HCV prevalence was lower in blood donors than in the GP, ranging from 0.2% (1992-1993) in Algeria to 1.7% (2005) in Yemen. The reporting quality of the outcomes in blood donors was good in the MENA countries, except in Qatar where no time framework was reported for the outcome. Some countries had anti-HCV prevalence estimates for children, transfused patients, contacts of HCV-infected patients, prisoners, sex workers, and men who have sex with men.

CONCLUSION
A substantial proportion of the reported outcomes may not help policymakers to develop micro-elimination strategies with precise HCV infection prevention and treatment programs in the region, as nowcasting HCV epidemiology using these data is potentially difficult. In addition to providing accurate information on HCV epidemiology, outcomes should also demonstrate practical and clinical significance and relevance. Based on the available data, most countries in the region have low to moderate anti-HCV prevalence. To achieve HCV elimination by 2030, up-to-date, good quality data on HCV epidemiology are required for the GP and key populations such as people who inject drugs and men who have sex with men.

Key words: Hepatitis C; Meta-research; Risk factors; Incidence; Genotype; Middle East and North Africa; Systematic review; Micro-elimination; Pakistan; Gulf Cooperation Council

Core tip: To achieve hepatitis C virus (HCV) infection elimination goals by 2030, precise prevention strategies targeting specific populations at higher risk of acquiring HCV infection and treatment programs require the development of evidence-based health policies. HCV infection epidemiology in the countries of the Middle East and North Africa was characterized in 37 systematic reviews (SR) during the last decade. Our systematic overview critically analyzes and synthesizes the findings of these SRs to map the evidence gaps in the region. Additionally, we assessed the quality of the reported outcomes and documented conflicts of interest of the SR authors who disclosed financial relationships with pharmaceuticals.

INTRODUCTION

In 2017, the World Health Organization estimated that 71 million people suffer from chronic hepatitis C virus (HCV) infection worldwide[1]. In 2015, HCV infection resulted in 399000 deaths[1], predominantly from complications such as liver cirrhosis and hepatocellular carcinoma (HCC)[1,2]. Egypt had the largest iatrogenic transmission of blood-borne pathogens in the general population (GP) worldwide[3]. The use of parenteral anti-schistosomiasis therapy in Egypt, extensively practiced with poor sterile techniques since the 1920s, is considered to be the leading cause of the dramatic increase in the human HCV reservoir in the GP[3]. In Egypt, HCV antibody (anti-) prevalence was estimated to be 15% in 2008[4] and 6% in 2014[5]. Globally, Pakistan has the second largest number of people with viremic HCV infection, after China[6]. Due to the high burden of HCV infection in Egypt and Pakistan, the MENA region has been identified as the region most affected region by HCV worldwide.

Our primary objective was to assess the quality of the data reported by the published systematic reviews (SRs) of HCV epidemiology in MENA countries taking into account conflict of interest disclosed by the authors of these SRs. Secondarily, we aimed to critically analyze all data reported by the SRs to map evidence gaps and to report a comprehensive synthesis.

MATERIALS AND METHODS

We conducted an overview of SRs based on the Cochrane

Handbook for Systematic Reviews of Interventions[7]. This overview is part of the Population Health Publications Assessment Project that aims to assess the methodological quality of published SRs on population health issues in the MENA region. The ultimate goal of this meta-research (*i.e.,* research on research[8]) is to evaluate and contribute to the improvement of population health research practices in the MENA region[9].

An *a priori* protocol was registered with the International Prospective Register of Systematic Reviews (PROSPERO registration number CRD42017076736)[10]. We report this overview according to the standards set out in the preferred reporting items for syste-matic reviews and meta-analyses (PRISMA) 2009 guidelines[11-13]. PRISMA checklists for SRs[11,12] and abstracts[13] are reported as Supplementary Materials 1 and 2, respectively. We also assessed the methodological quality of our overview using the Assessment of Multiple Systematic Reviews (AMSTAR) tool[14]. The AMSTAR checklist is provided as Supplementary Material 3.

Inclusion and exclusion criteria
We included all SRs (publication date: January 2008 to December 2016) of HCV infection related to the countries in the MENA region published in peer-reviewed journals since 2008, the publication year of the first version of the Cochrane Handbook for Systematic Reviews of Interventions[7]. We included MENA countries where Arabic, English, French, and/or Urdu are the primary official languages and/or the medium of instruction in the colleges/universities. These languages are those spoken by the authors of this overview.

Population of interest
Our population of interest was the population living in the following 20 countries: Algeria, Bahrain, Djibouti, Egypt, Iraq, Jordan, Kuwait, Lebanon, Libya, Morocco, Oman, Pakistan, Palestine, Qatar, Saudi Arabia, Sudan, Syria, Tunisia, the United Arab Emirates (UAE), and Yemen. These selected MENA countries have a combined population of over 560 million people, accounting for approximately 8% of the world's population[15]. We excluded SRs of HCV infection in populations from countries outside the MENA region and SRs with military personnel who were based in the MENA region but not from the region.

Primary outcomes
We extracted the following variables: Anti-HCV prevalence and incidence in different at-risk populations, HCV viremic rates in HCV positive individuals, and HCV viremic prevalence in the GP. Viremic infection represents the presence of HCV in the bloodstream detected with polymerase chain reaction (HCV RNA-positive). We also extracted the prevalence of co-infection with HBV (hepatitis B surface antigen, HBsAg), human immunodeficiency virus (HIV), or schistosomiasis; HCV genotype/subtype distributions; and risk factors for

HCV transmission. Additionally, we extracted conflicts of interest reported by the SR authors.

Literature search and data management
Two reviewers (AA and HA) independently and systematically searched the Medical Literature Analysis and Retrieval System Online (MEDLINE) through the search engine PubMed[16] using broad search criteria (Supplementary Material 4). The search criteria, limited to reviews, systematic reviews, and meta-analysis, identified 5758 reviews (Figure 1). After removing duplicate publications of the same reviews with Endnote[17], title/abstract screening and then full-text screening were conducted by AA and HA with Rayyan software[18,19]. Discrepant inclusions of SRs were discussed by AA, HA, and KC under the supervision of the senior authors. We eventually included 37 SRs of HCV infection in MENA countries (Figure 1). The characteristics of these included SRs are described in Supplementary Material 5. Data extraction was conducted by AA and KC and crosschecked for accuracy.

Conflicts of interest
We recorded the conflicts of interest reported by the SR authors who disclosed financial relationships. An asterisk (*) was inserted next to the outcomes reported by SRs with at least one author who disclosed direct or indirect financial relationships with Gilead Science, AbbVie, Merck, Bristol-Myers Squibb, or Janssen Therapeutics. In 2013, Gilead Science launched sofosbuvir-based regimens to cure chronic infections with HCV genotypes 1, 2, 3, and 4[20] (viremic cases) that made elimination of HCV infection achievable for the first time. A large proportion of patients (> 90%) treated with sofosbuvir associated with another direct-acting antiviral drug (DAA) demonstrated a sustained virological response[21]. The recommended regimens and treatment durations differ according to the HCV genotype infection. Additionally, sofosbuvir-based regimens appear to be relatively safe[21]. However, cases of cardiac events have been reported when sofosbuvir-based treatments were administered in combination with amiodarone and/or propranolol[22,23]. Since then, new DAAs have been developed by other pharmaceuticals (AbbVie, Merck, Bristol-Myers Squibb, and Janssen Therapeutics)[24].

Quality assessment and conflict of interest
Good quality outcomes reported by the SRs were defined as having the population, outcome, timing, and setting defined as recommended by the PICOTS framework[25,26] (intervention and comparator being irrelevant in our overview) and a sample size > 100[27]. A maximum score of six was given to the outcome if the following measures were included: (1) Population profile, such as pregnant women and patients under hemodialysis; (2) the method used for the outcome estimation, such as meta-analysis or weighted average; (3) the data collection period; (4) the study geographic location; (5) the number of studies;
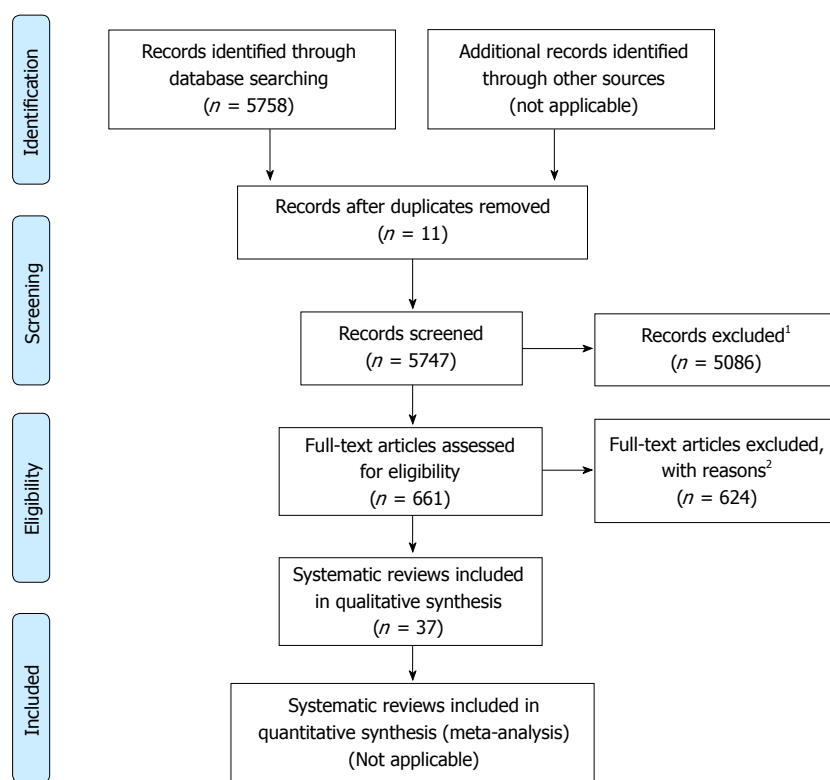
Figure 1 PRISMA 2009 Flow diagram describing the selection of the systematic reviews reporting hepatitis C outcomes in our systematic overview. Reasons of exclusion: [1]Systematic reviews were excluded from the overview because they did not meet our inclusion criteria; [2]Not a systematic review (*n* = 116), no hepatitis C outcomes (*n* = 472), not a polulation from MENA (*n* = 34), duplicate (*n* = 1), and publication in Portuguese (*n* = 1).

and (6) a total sample size > 100.

Synthesis
We narratively synthesized the data on HCV infection in each of the 20 MENA countries in at-risk populations. We defined the GP as individuals from the community who are not in any specific population category such as blood donors or outpatients. More specifically, we reported HCV epidemiology in the GP separately from that in blood donors. Blood donors are selected healthy populations that may not be representative of the GP[28,29]. Therefore, if a SR reported an outcome for the GP including specific population categories, we reported this GP specifying all the included population categories.

Extracted data from the included SRs were compiled into evidence tables. We reported anti-HCV prevalence in at-risk populations from the SRs and the quality assessment of the reported prevalence (Supplementary Materials 6 and 7). Country-level anti-HCV prevalence was classified into low (< 1.5%), moderate (1.5%-3.5%), and high (> 3.5%) levels[30]. If available, we also reported the viremic rate in anti-HCV positive individuals and the viremic prevalence in the GP.

For each country, we mapped evidence gaps with the most up-to-date anti-HCV prevalence with the best quality assessment (highest quality assessment score) in the GP; blood donors; pregnant women; children; patients on dialysis; hemophiliac, thalassemic, and transfused patients; patients with HCC, acute liver disease, chronic liver disease (CLD), HIV and/or other

sexually transmitted infections; healthcare workers; barbers; contacts of HCV-infected patients; people who inject drugs (PWID); prisoners; sex workers; and men who have sex with men (MSM) (Tables 1-3). We took financial disclosures into account when assessing and reporting these outcomes[31]. Whenever two outcomes describing the same population in the same country had the same quality score, we reported the outcome extracted from the SR the authors of which disclosed no financial relationship with HCV DAA pharmaceutical companies.

RESULTS

Quality assessment - PICOTS framework
Only six out of 37 SRs of HCV epidemiology in the MENA region published after 2008 conducted a quality assessment of the studies included[6,27,32-35]. HCV prevalence or incidence is meaningful only when the population, the outcome, the study setting, and the study period are stated (PICOTS framework)[25]. Additionally, for SRs, reporting the quantitative synthesis method used and the number of studies included in the synthesis as recommended by PRISMA guidelines[11-13] is critical to enable the assessment of the quality of the outcome (Tables 1-3). Some SRs failed to precisely report the methodology they used for their quantitative synthesis[36-38].

In addition to providing a time framework for morbidity measurements, reporting the collection period for HCV prevalence is essential because changes in

Table 1 Hepatitis C antibody prevalence evidence gap mapping with quality assessment, North Africa and Pakistan

	Algeria	Djibouti	Egypt	Libya	Morocco	Sudan	Tunisia	Pakistan
Blood donors	0.2% (1992-1993)[1]	0.3% (1998-2000)[1]	1.7%-16.8% (2006-2009)[1]	0.9%-6.6% (1992-2001)[1]	0.2%-0.8% (2005-2011)[1]	0.0%-1.3% (2005-2007)[1]	0.1%-0.9% (2000-2010)[1]	0.2%-8.6% (2000-2006)[1]
Pregnant women	0.6% (2008)[1]	No data	8.6%-12.7% (-)[3]	0.4%-2.3% (-)[3]	1.0% (-)[2]	0.6% (2006)[1]	0.2% (2006)[1]	5.3% (2001-2006)[1]
Children	No data	No data	2.1%-5.8% (2002-2005)[1]	No data	No data	No data	No data	1.7% (-)[2]
Hemodialysis, renal dialysis, and/or dialysis patients	39.0% (-)[3]	No data	46.1%-100% (2002-2009)[1]	32.3% (2009-2010)[1]	54.1%-68.3% (2003-2004)[1]	8.5%-23.7% (2005-2010)[1]	14.6%-32.6% (2000-2002)[1]	38.8% (2003-2005)[1]
Hemophiliac or other bleeding disorder patients	30.0% (2010-2011)[2]	No data	No data	No data	2.3%-42.4% (1981-2006)[1]	13.0% (2008)[2]	55.7% (-)[2]	25.0%-56.0% (2000-2002)[1]
Transfused patients	No data	No data	11.1%-81.6% (2000-2008)[1]	10.8% (1999-2001)[1]	No data	No data	4.7% (2008-2009)[1]	13.2%-15.4% (2002)[1]
Thalassemic patients	No data	No data	19.5%-69.6% (2000-2010)[1]	No data	68.3%-76.0% (-)[3]	No data	6.1% (-)[2]	36.2-56.8% (1999-2003)[1]
Patients with hepatocellular carcinoma	No data	No data	85.9% (1997-2004)[1]	No data	57.3% (2003-2006)[2]	11.0% (1996-1998)[1]	19.0%-63.5% (1982-< 1995)[2]	53.7% (-)[2]
Patients with acute liver disease	No data	No data	No data	No data	0.0% (1999)[1]	6.3% (2007)[2]	8.0%-48.0% (< 1995)[3]	No data
Patients with chronic liver disease	No data	No data	No data	No data	73.7% (1983-1986)[2]	No data	80.5% (2005-2008)[2]	0.0%-86.6% (2000-2005)[1]
Healthcare workers	No data	No data	No data	2.0%-6.8% (1992-2001)[1]	No data	0.0% (2005-2007)[1]	1.0% (2005)[1]	5.6%-6.0% (2001-2002)[1]
Barbers	No data	No data	12.3% (-)[3]	No data	1.1%-5.0% (2001-2007)[1]	No data	No data	No data
Contact of patients with hepatitis C	No data	No data	No data	No data	No data	No data	No data	4.3%-38.0% (2000-2004)[1]
People who inject drugs	No data	No data	63.0% (-)[2]	94.2% (2010)[1]	22.9%-79.2% (2010-2012)[1]	No data	21.7%-29.1% (2009-2012)[1]	57.0% (-)[2]
Prisoners	No data	No data	No data	23.7% (2006)[1]	No data	No data	No data	No data
Patients with HIV and/or other sexually transmitted infection	No data	No data	No data	44.9%-54.1% (2003)[1]	5.4% (2006-2010)[1]	1.7% (2010-2012)[1]	35.5% (1997-2006)[1]	No data
Sex workers	No data	No data	No data	5.2%-7.3% (2010-2011)[1]	No data	0.0%-5.1% (2011-2012)[1]	1.1% (2007)[1]	No data
Men who have sex with men	No data	No data	No data	No data	No data	0.0%-5.9% (2011-2012)[1]	No data	No data

Prevalence, year of data collection. [1]Good quality; [2]Average quality; [3]Poor quality.

risk factors for HCV were demonstrated over time in Egypt and other countries[39]. Out of the 29 SRs reporting the anti-HCV prevalence, 16 (55%) failed to accurately report the years during which data collection occurred[32,34,36-38,40-50]. Thirteen SRs did not report the data collection years at all[34,36-38,40-48], two reported the publication year as or instead of the data collection period of the study[32,49], and one SR published in 2011 stated "unless indicated the estimates were for 2004 because of lack of more recent data"[50]. The choice of this year, *i.e.,* 2004, was not justified and appears arbitrary. For instance, the authors reported an anti-HCV prevalence in individuals with sexually transmitted diseases (STD) in Saudi Arabia of 15.9%* in 2004, as no year was indicated[50]. Remarkably, the actual study that estimated the prevalence in individuals with STDs in Saudi Arabia reported 1990 as the year of data collection[51]. Additionally, we identified SRs pooling the prevalence measured over a long time period such as a pooled estimate for 1982-2010 in Tunisia[52] or for 1989-2012 in Sudan[27]. These estimates that encompass long time periods do not reflect changes in prevalence over time and do not accurately describe the past and current HCV epidemiology in a population.

Similarly, for genotype distribution, only two[50,53] of the 18 SRs[6,27,33,37,38,40,43-45,48-50,53-58] reported HCV genotype/subtype distributions, and one[50] of the 12 SRs[27,39,40,44,45,50,52,53,57,59-61] reported risk factors with the data collection year provided, which would be an indicator if changes in genotype/subtype geographical distributions or in risk factors occurred over time. However, regarding incidence, six SRs[33,48,50,52,59,61] identified data points, and all of them reported the data collection years as those provided by the included studies.

Regarding the definitions of populations, we identified SRs pooling anti-HCV prevalences from populations at differing risk levels for acquiring HCV infections[27,33,37,41,52,59,62,63]. We question the meaningfulness of these estimated outcomes for mixed populations. How would clinicians and policymakers interpret and use an estimated anti-HCV prevalence for a mixed population including, for instance, patients with lymphoproliferative disorders, prisoners, patients with depression, mothers, and sex workers[41]? Regarding the GP, we identified SRs that combined blood donors with the GP[27,33,52,59,62]. Healthy blood donors (voluntary and non-remunerated)

Table 2 Hepatitis C antibody prevalence evidence gap mapping with quality assessment, Arab Peninsula						
	Bahrain	**Kuwait**	**Oman**	**Qatar**	**Saudi Arabia**	**United Arab Emirates**	**Yemen**
Blood donors	No data	0.8%-1.2% (nationals) (2002) 5.4%-13.5% (migrants) (2002)[1]	0.4%-0.7% (2006-2011)[1]	1.1% (nationals and migrants) 0.5% (nationals) (-)[2]	0.0%-5.6% (nationals and migrants, 1988-2009) 0.4%-4.2% (nationals, 1990-2009) 0.0%-34.0% (migrant, 1990-2002)[1]	0.5%-1.1% (2004-2009)[1]	1.7% (2005)[1]
Pregnant women	No data	No data	No data	No data	0.1%-4.6% (1989-2002)[1]	13.0%-13.4% (migrants) (1994-1996)[1]	3.3%-8.5% (2011)[1]
Children	No data	No data	No data	No data	0.7%-1.8% (1989-2002)[1]	No data	2.1% (2007-2009)[1]
Hemodialysis, renal dialysis, and/or dialysis patients	9.2% (-)[3]	27.0%-71.0% (1992-1995)[1]	26.5% (1991)[1]	44.6% (-)[2]	14.7%-78.2% (1995-2008)[1]	24.4% (1991-1993)[1]	40.0%-62.7% (1997-2013)[1]
Hemophiliac or other bleeding disorder patients	60.0% (1992)[2]	No data	No data	No data	78.6% (-)[3]	No data	No data
Transfused patients	No data	No data	No data	No data	4.6%-78.6% (1990-2011)[1]	No data	No data
Thalassemic patients	No data	33.0% (1965-1995)[1]	No data	No data	12.7%-70.0% (1990-2000)[1]	18.8% (1990-2001)[2]	No data
Patients with hepatocellular carcinoma	No data	No data	No data	No data	48.5% (2006-2008)[1]	No data	28.4%-38.2% (2001-2010)[1]
Patients with acute liver disease	No data	No data	No data	No data	3.4%-40.7% (2000-2005)[1]	1.2% (2006-2007)[1]	6.4%-8.8% (1997-1999)[1]
Patients with chronic liver disease	No data	37.8% (-)[2]	No data	29.4 (2000-2005)[1]	11.9%-65.0% (1989-2004)[1]	43.7% (-)[2]	33.8% (-)[3]
Healthcare workers	No data	0.9% (-)[2]	0.0% (-)[2]	No data	0.0%-0.3% (2001-2005)[1]	No data	1.1%-3.5% (-)[3]
Barbers	No data	No data	No data	No data	No data	No data	No data
Contact of patients with hepatitis C	No data	No data	No data	No data	0.0%-1.6% (-)[2]	27.0% (1994-1996)[1]	No data
People who inject drugs	No data	No data	48.1% (2011)[1]	No data	14.4%-54.7% (1995-2004)[1]	No data	No data
Prisoners	No data	No data	No data	No data	No data	No data	No data
Patients with HIV and/or other sexually transmitted infection	No data	0.9% (2012)[1]	No data	No data	12.0%-15.9% (1985-2010)[1]	No data	No data
Sex workers	No data	No data	No data	No data	No data	No data	No data
Men who have sex with men	No data	No data	No data	No data	No data	No data	No data

Prevalence, year of data collection. [1]Good quality; [2]Average quality; [3]Poor quality.

recruited with selection programs likely have lower anti-HCV prevalence than the GP[64]. Hence, in SRs pooling blood donor estimates with the GP estimates, the pooled estimates likely underestimate the actual anti-HCV prevalence in the GP. Furthermore, as meta-analyses give greater weight to studies with higher sample sizes and the sample sizes of studies describing anti-HCV prevalence in blood donors are often large, these pooled anti-HCV prevalences are more likely to be underestimated. In a country such as Djibouti where there were no identified studies reporting in the GP and only one identified study reporting on blood donors, reporting the estimate in blood donors as the lower limit of possible anti-HCV prevalence in the GP[6] might be relevant because of the scarcity of data. Riou *et al*[35] used that estimate to provide an adjusted estimate for the GP. We believe that in countries where data points among the GP are available, blood donors should be excluded.

Similarly, for reported genotype/subtype distributions and risk factors, mixing populations limited the ability to analyze that data in-depth.

Conflict of interest
Out of the 37 SRs included in our overview, 32 SRs had authors who disclosed no conflict of interest. In one SR[65], authors disclosed financial support from a US National Science Foundation Graduate Research Fellowship, a Sigma Xi Grant-in-Aid of Research, and the Global Health Program at the University of Michigan School of Public Health. Four SRs[6,34,50,56] reported at least one author who disclosed a direct or indirect financial relationship with pharmaceutical companies; among these SRs, three reported financial support from HCV DAA pharmaceutical companies[6,50,56]. Gower *et al*[6] reported indirect financial support from Gilead Science,

Table 3 Hepatitis C antibody prevalence evidence gap mapping with quality assessment, Fertile Crescent region

	Iraq	Jordan	Lebanon	Palestine	Syria
General population	0.0%-4.0 % (2004-2012)[1]	No data	0.6%-2.9% (-)[2]	No data	2.8% (2004)[1]
Blood donors	0.2%-2.8% (2003-2013)[1]	0.1%-0.9% (2003-2011)[1]	0.0%-3.4% (< 2003)[1]	0.2%-0.3% (2003-2013)[1]	0.4% (2000-2011)[1]
Pregnant women	0.0%-5.1% (2004-2010)[1]	No data	No data	No data	No data
Children	0.0% (2007-2009)[1]	No data	No data	No data	No data
Hemodialysis, renal dialysis, and/or dialysis patients	4.9%-42.6% (2002-2011)[1]	21.0%-49.8% (2003-2008)[1]	16.0%-27.0% (-)[2]	17.9%-27.4% (2007-2013)[1]	21.0% (2006)[1]
Hemophiliac or other bleeding disorder patients	6.7%-40.3% (2006-2012)[1]	No data	10.0% (-)[3]	No data	20.5% (2007-2011)[1]
Transfused patients	3.4%-4.5% (2004-2008)[1]	No data	No data	No data	13.3% (2000)[2]
Thalassemic patients	4.0%-46.0% (2003-2012)[1]	32.8% (2008)[1]	0.0%-14.0% (> 1999)[1]	No data	No data
Patients with hepatocellular carcinoma	26.1% (2000-2003)[2]	No data	19.6% (1998-2003)[2]	No data	No data
Patients with acute liver disease	0.0%-71.9% (2007-2011)[1]	No data	No data	No data	1.0%(1995-1998)[1]
Patients with chronic liver disease	3.8%-62.0% (2005-2009)[1]	No data	No data	No data	No data
Healthcare workers	0.0%-9.1% (2002-2010)[1]	0.7% (1999)[1]	0.4% (1999)[1]	No data	3.0%-3.8% (-)[2]
Barbers	0.3% (1999-2001)[1]	No data	No data	No data	No data
Contact of patients with hepatitis C	1.2%-1.4% (1996-2001)[1]	No data	No data	No data	No data
People who inject drugs	No data	No data	52.8% (2007-2008)[1]	45.2% (2010)[1]	60.5% (-)[3]
Prisoners	0.6% (1996-2001)[1]	No data	3.4% (-)[3]	No data	No data
Patients with HIV and/or other sexually transmitted infection	1.2% (-)[2]	No data	7.7% (-)[3]	No data	No data
Sex workers	No data	No data	0.0% (2007-2008)[1]	No data	2.0% (-)[2]
Men who have sex with men	No data	No data	0.0% (2007-2008)[1]	No data	No data

Prevalence, year of data collection. [1]Good quality; [2]Average quality; [3]Poor quality.

AbbVie, and Bristol-Myers Squibb. Bruggmann *et al*[56] and Sievert *et al*[50] reported financial support from Gilead Science, AbbVie, Merck, Bristol-Myers Squibb, and Janssen Therapeutics. Bruggmann *et al*[56] had 34 authors who disclosed direct or indirect financial relationships with at least one of the HCV DAA pharmaceutical companies (37% of the total number of authors). Furthermore, six authors did not disclose their conflict of interest. In Sievert *et al*[50], out of the 29 authors, 13 (45% of the total number of authors) disclosed direct or indirect financial relationships with at least one of the HCV DAA pharmaceuticals.

Interestingly, two[6,56] of the three SRs that reported financial support from HCV DAA pharmaceutical companies were published in 2014 and were the only ones that estimated the total number of viremic cases in the GP. They were also the only SRs that estimated the genotype/subtype distributions in the GP of the MENA region, except for Morocco[43], Pakistan[50,55], and Tunisia[43] where the distributions were also computed by a couple of additional SRs. In Saudi Arabia, the third SR estimating genotype/subtype distributions also had authors who disclosed financial relationships with HCV DAA pharmaceutical companies[50]. Remarkably, in both SRs[6,56], the authors did not follow the PRISMA guidelines (2009)[11] for reporting their reviews in detail; thus, we question the replicability of the reviews. Flow diagrams depicting the numbers of records identified,

included, excluded, and, most importantly, the reasons for the exclusions were not presented in those SRs[6,56]. Bruggmann *et al*[56] did not report the search criteria, eligibility criteria, or the risk of bias assessment of the included studies. Additionally, Bruggmann *et al*[56] reported that an expert panel reviewed the findings and analysis, and Gower *et al*[6] reported including "personal communication with experts within countries". However, the authors did not report how they defined "expert" or any conflicts of interest of these experts[6,56]. These reviews[6,56] had been cited 185[56] and 890[6] times, respectively, by February 12, 2018 (Google Scholar citations) and published in journals with impact factors of 3.9 and 11.3 (in 2014), respectively.

HCV prevalence
In the GP, the estimations of the most up-to-date anti-HCV prevalence with the best quality ranged widely across MENA countries (Figure 2 and Tables 1-3). We identified low prevalence (< 1.5%) in Djibouti, Kuwait, Oman, Qatar, and UAE; moderate prevalence (1.5%-3.5%) in Algeria, Iraq, Lebanon, Libya, Morocco, Saudi Arabia, Sudan, Syria, and Tunisia; and high prevalence (> 3.5%) in Egypt, Pakistan, and Yemen. In Pakistan, anti-HCV prevalence may have increased from 5.4% in 1996-2007[62] to 6.7%* in 2007-2008[6]. No reported data in the GP was identified for Bahrain, Jordan, or Palestine.
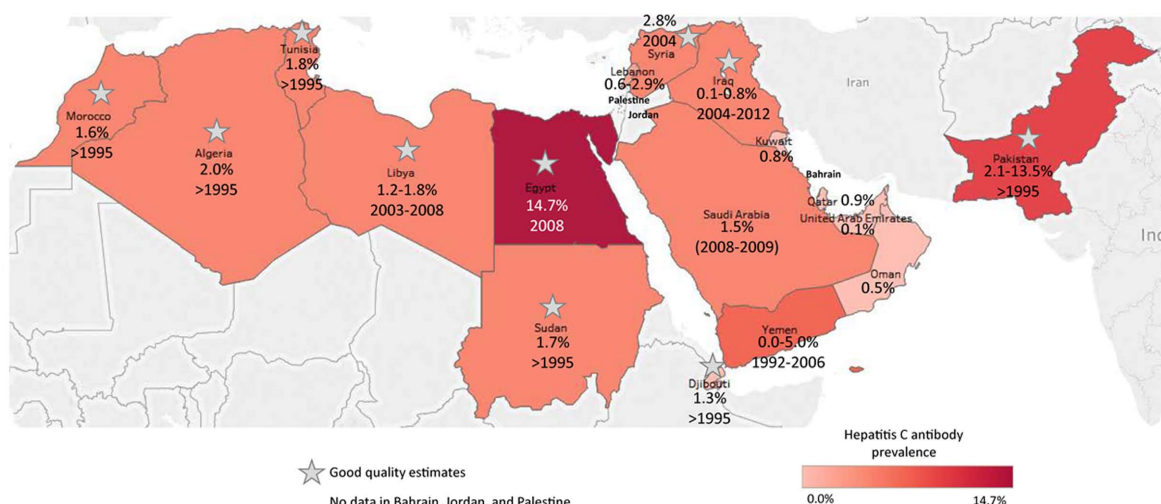
Figure 2 Hepatitis C antibody prevalence evidence gap mapping with quality assessment, general population, MENA. MENA: Middle East and North Africa.

Summary points pertaining to the estimated anti-HCV prevalences in blood donors, pregnant women, children, and patients with acute liver disease or CLD and HCC are highlighted in Supplementary Materials 8.

We identified that HCV transmission most likely occurs in healthcare settings. In all 20 MENA countries, patients with blood disorders such as hemophilia and thalassemia, patients undergoing hemodialysis, and patients who received transfusions had higher prevalences of anti-HCV than the prevalence in the GP. This suggests that transfusion-associated HCV infection may have occurred in these patients (Supplementary Material 8). Additionally, anti-HCV prevalences in healthcare workers in Iraq, Libya, and Syria suggest that in these countries, HCV transmission may also be due to occupational exposure (Supplementary Material 8). Community-acquired HCV infection may also occur in the MENA countries; however, we identified evidence gaps in the region (Supplementary Material 8).

Anti-HCV prevalence measures were not often identified for key populations such as PWID, prisoners, and MSM. Anti-HCV prevalence data were predominantly missing for the countries of the Arab Peninsula. No reported data were identified for MSM, prisoners, or sex workers in Bahrain, Kuwait, Oman, Qatar, Saudi Arabia, the UAE, or Yemen. For PWID, anti-HCV prevalence data were identified in Egypt, Lebanon, Libya, Morocco, Oman, Pakistan, Palestine, Saudi Arabia, Syria, and Tunisia. For prisoners, reported anti-HCV prevalence data were identified in Iraq, Lebanon, and Libya.

Sexual transmission may also be a cause of HCV infection in some MENA countries. Anti-HCV prevalence data in patients with HIV or other STDs were missing for Algeria, Bahrain, Djibouti, Egypt, Jordan, Oman, Pakistan, Palestine, Qatar, Syria, the UAE, and Yemen. Anti-HCV prevalences in sex workers were reported for a few countries.

HCV incidence
The incidence rate reflects the status of HCV transmission in a population. Nevertheless, up-to-date incidence data in MENA countries were lacking (Tables 4 and 5). In the GP, the anti-HCV incidence rate was reported only in Egypt, at 0.8-6.8 per 1000 person-year in 1997-2003. The incidence rate was the highest in patients undergoing hemodialysis in the UAE, at 108 per 1000 person-year in 2003-2004. The incidence rates in patients undergoing hemodialysis were reported for Iraq, Jordan, Morocco, Tunisia, and the UAE. These findings suggest ongoing HCV transmission within healthcare settings in these countries.

Viremic rate
In the GP, the HCV viremic rate among anti-HCV positive individuals was 51.6%* (2011) in Saudi Arabia[6]; approximatively 70%* in Egypt (2008)[6], Morocco (2005-2011)[6], and the UAE (study dates not reported)[6]; 80.0%* (2012) in Tunisia[6]; and 87.4%* (2007-2008) in Pakistan[6]. Country-level HCV viremic prevalences ranged between 0.7%* [95% confidence interval (CI): 0.3%-3.8%, 2011][6] in Saudi Arabia and 10.0%* (CI: 7.0%-12.2%, 2008)[6] in Egypt. The viremic prevalence was 1.1%* (0.4%-1.4%, 2005-2011)[6] in Morocco, 1.0%* (0.2%-2.0%, 2012)[52] in Tunisia, and 5.8%* (1.4%-8.7%, 2007-2008)[6] in Pakistan. In anti-HCV positive blood donors, the viremic rate was 60.0% in Libya (1992-2001)[52] and 20.0% in Saudi Arabia (1988-2009)[59]. In healthcare workers, the viremic rate was reported in Libya as 69.2% (1992-2004)[52]. In dialysis patients, the HCV viremic rate was 51.0% in Tunisia before 2000[52] and 60.5%-93.3% in 2000-2003[52]; in Libya, it was 72.0% (before 2001)[52]. In HIV-infected patients in Morocco, the viremic rate was reported as 70.4% (study dates not reported)[49].

Co-infection with hepatitis B virus, HIV, or schistosomiasis
We identified two SRs[36,47] that reported HBV (HBsAg) and anti-HCV co-infection prevalence estimates; these estimates were only in HCC patients, and neither of

Table 4 Hepatitis C antibody incidence and/or seroconversion risk rate relative to the total sample size, and evidence gap mapping, Middle East and North Africa

Country	Systematic review	Literature search period	Number of studies identified	Number of subjects	Population type	Estimation time period	HCV incidence rate (per 1000 person-year)	Loss of follow-up	HCV sero-conversion risk (%)	Duration of follow-up
Algeria	Fadlalla, 2015[52]	1980-Unknown	0	0						
Libya	Fadlalla, 2015[52]	1980-Unknown	0	0						
Morocco	Fadlalla, 2015[52]	1980-Unknown	1	303	Hemodialysis patients	2003-2004	97	-	-	-
Tunisia	Fadlalla, 2015[52]	1980-Unknown	1	276	Hemodialysis patients	2000-2002	28	-	-	-
Djibouti	Chaabna, 2016[27]	Inception-2015	0	0						
Sudan	Chaabna, 2016[27]	Inception-2015	0	0						
Egypt	Hussein, 2016[48]	Inception-Unknown	-	-	-	-	6.9	-	-	-
	Mohamoud, 2013[61]	Inception-Unknown	2	2852	Children of villages with high HCV prevalence	2000-2006	2.7	-	-	-
	Mohamoud, 2013[61]	Inception-Unknown	3	10318	General population	1997-2003	0.8-6.8	-	-	-
	Mohamoud, 2013[61]	Inception-Unknown	1	2177	Pregnant women	1997-2006	5.2	-	-	-
	Sievert, 2011[50]	Unknown	-	-	Pregnant women	-	5.2	-	-	-
Pakistan	Sievert, 2011[50]	Unknown	0	0						

these SRs[36,47] reported study dates/years. In Egypt, Pakistan, Saudi Arabia, and Tunisia, the anti-HCV prevalence was higher than the HBsAg prevalence, while the opposite finding was observed in Lebanon, Sudan, and Yemen (Supplementary Materials 9).

In Libya, 90.1% (CI: 78.2%-100%) of HIV-infected prisoners had an HCV co-infection (study dates not reported)[49]. In HIV-infected children, the prevalence of HCV co-infection was 43.0%-46.0% (1998-1999)[52]. The most up-to-date estimates of HCV co-infection in HIV-infected patients were 5.4% (2006-2010) in Morocco[52], 1.7% (CI: 0.2%-2.3%, study date not reported) in Sudan[27], 33.5% (CI: 20.4%-46.6%, 1997-2005) in Tunisia[49,52], and 7.7% (study dates not reported) in Lebanon[33].

Studies on schistosomiasis and HCV co-infection were reported for Egypt[45,61], Sudan[27,45,46], and Saudi Arabia[59]. In Egypt, 7.7%-84.0% (2002-2011) of the schistosomiasis patients were also infected by HCV[61]. Prior to 2000, HCV prevalence in schistosomiasis patients was 16.3%-42.5%[61]. In Sudan, the prevalence of schistosomiasis and HCV co-infection ranged from 4.5%[27] to 31.0% in 2001[27]. In Saudi Arabia, 17.9% of schistosomiasis patients were also infected with HCV[59].

HCV genotype and subtype
In the GP, genotype 1 was the most common genotype in Algeria (69.2%*)[6], Morocco (75.9%)[6,43], Tunisia (84.0%)[6,43], and Jordan (73.3%*)[6], while in Pakistan the most common genotype was genotype 3 (67.5%-86.7%)[40].

No data in the GP was reported for Bahrain, Djibouti, Oman, Qatar, or Yemen. In the other MENA countries it was genotype 4 (45.2*-93.1%*)[6,43,50,54,56] (Supplementary Materials 10 and 11). Interestingly, in Jordan, systematic reviews reporting genotype 4 as the most common genotype in the country had pooled estimates from the GP and other at-risk populations. In Morocco and Tunisia, subtype 1b was the most common genotype in the GP[43], while in Pakistan[50,55] and Saudi Arabia[50], it was 3a. In pregnant women, genotype 4 was reported as the most common genotype in Algeria, Egypt, Iraq, Libya, Saudi Arabia, Sudan, Tunisia, and Yemen[44], whereas genotype 1 was the most common genotype in Morocco[44]. Genotype distributions in blood donors[38] and in clinical populations, such as patients with CLD, non-Hodgkin lymphoma, thalassemia, or hemophilia, were similar to the distributions identified in the general population[38,57]. However, in PWID in Saudi Arabia, genotype 1 was reported as the most common genotype (48%), followed by genotype 3 (36.0%*)[50], whereas in Lebanon, genotype 3 was the most common genotype (26.3%-57.0%), followed by genotype 1 (21.0%-42.1%) and genotype 4 (18.0-34.6)[38].

Risk factors associated with HCV infection
The risk factors for acquiring an HCV infection reported for MENA countries were factors associated with medical practices (blood transfusion, catheter use, hemodialysis, nosocomial exposure, and invasive procedures, among others)[27,39,40,44,45,50,52,53,57,59-61] (Supplementary Material 12).

Table 5 Hepatitis C antibody incidence and/or sero-conversion risk rate relative to the total sample size, and evidence gap mapping, Middle East and North Africa

Country	Systematic review	Literature search period	Number of studies identified	Number of subjects	Population type	Estimation time period	HCV incidence rate (per 1000 person-year)	Loss of follow-up	HCV sero-conversion risk (%)	Duration of follow-up
Iraq	Chemaitelly, 2015[33]	1985-2015	1	57	Hemodialysis patients	2009	-	0	40.3	12 mo
	Chemaitelly, 2015[33]	1985-2015	1	123	Pediatric patients with acute lymphoblastic leukemia	2007	-	0	3.2	30 mo (median)
	Chemaitelly, 2015[33]	1985-2015	1	26	Newborn to HCV infected women	-	-	0	0	6 mo
	Chemaitelly, 2015[33]	1985-2015	1	85	Pediatric cancer patients on chemotherapy	2006-2007	-	22	3.2	12 mo
	Chemaitelly, 2015[33]	1985-2015	1	60	Healthy children	2007-2009	-	0	0	6 mo
	Chemaitelly, 2015[33]	1985-2015	1	29	Pediatric patients with leukemia on chemotherapy	2007-2009	-	0	3.5	6 mo
	Chemaitelly, 2015[33]	1985-2015	1	27	Pediatric patients with leukemia who have had their baseline screening prior to chemotherapy	2007-2009	-	0	0	6 mo
Lebanon	Chemaitelly, 2015[33]	1985-2015	0	0						
Palestine	Chemaitelly, 2015[33]	1985-2015	0	0						
Jordan	Chemaitelly, 2015[33]	1985-2015	1	1300	Hemodialysis patients	2003	-	-	9.2	12 mo
Syria	Bashour, 2016[91]	Unknown	0	0						
	Sievert, 2011[50]	Unknown	0	0						
United Arab Emirates	Mohamoud, 2016[59]	Unknown	1	-	Hemodialysis patients	1995	108	-	-	-
Yemen	Chaabna, 2016[27]	Inception-2015	0	0						

These risk factors suggest that iatrogenic transmission is specifically found in Egypt[39,44,45,50,60,61], Iraq[44], Pakistan[40,50,53,57], Saudi Arabia[50], Sudan[44,45], Syria[50], and Yemen[44]. In Egypt, Pakistan, Saudi Arabia, Sudan, and Syria, factors associated with the community were also reported (contacts of HCV-infected patients, sharing razors, circumcision, injection from informal health provider, and tattooing, among others)[39,40,44,45,50,53,57,60,61]. Age[45,59,61], level of education in Yemen[44], intravenous drug use in Pakistan[57] and Syria[50], and homosexuality in Pakistan[57] were also associated with HCV infection.

DISCUSSION

Stretching from North Africa through the Middle East and into central Asia, the MENA region demonstrates a wide range of anti-HCV and viremic prevalences and diversity in HCV genotype distributions. For each country, our mapping of evidence gaps and quality assessment of outcomes reported on the available HCV morbidity measurements, their quality, and how well the SRs reported them and emphasized the opportunities for further population health research to better characterize HCV epidemiology in the region.

Quality assessment

Our overview of SRs identified that a substantial proportion of SRs on HCV epidemiology in MENA failed to report their outcomes following the PICOTS framework[25,26]. This can potentially lead to imprecise reported information that can, in turn, mislead data interpretation[26]. In addition to providing accurate information on HCV epidemiology, outcomes should also demonstrate practical and clinical significance and relevance. While most SRs of clinical research were determined to not be useful because their conclusions were false or exaggerated[66], it appears that we should also question the usefulness of SRs of population health research on HCV epidemiology in the MENA region. The SRs of HCV epidemiology in the MENA region stated that their aim was to provide a clear picture of the situation in the region. However, a substantial proportion of the

SRs in our overview lacked the key identified features of transparency ("are methods, data, and analyses verifiable and unbiased?"[66]) and pragmatism ("inferences should be applicable to real-life circumstances"[66]), which also are lacking in clinical research[66].

To reach the Global Health Sector Strategy on Viral Hepatitis (GHSS-VH) 2030 elimination goal[67], SRs of HCV epidemiology should assist policymakers in developing treatment programs and effective, precise prevention strategies for micro-elimination targeting specific populations at higher risk of acquiring HCV infections. Additionally, SRs of HCV epidemiology should guide changes to make clinical practices safer. Hence, an accurate picture is not obtainable by pooling population estimates of groups at different risk of acquiring HCV infections, such as patients undergoing hemodialysis combined with PWID, MSM combined with healthcare workers, or the GP combined with blood donors. Furthermore, estimated HCV outcomes should be as up-to-date as is reasonably possible, encompassing a narrow interval of time to attempt to depict the current situation in the region. The outcomes can be pooled by period, so potential changes in the epidemiological profile can be demonstrated. As such, dissemination of pooled estimates that combine data from many studies, some conducted two decades ago, in order to increase the statistical power does not appear to be useful in nowcasting the epidemiology of HCV in the region and is not helpful in developing effective preventive and treatment programs essential to attaining the 2030 HCV infection elimination goal.

Conflict of interest
The evaluation of SRs should not be limited to the assessment of the reported methods, assumptions, data, and results[31]. Our overview emphasizes the significance of considering the disclosure of financial relationships while assessing research[31]. Researchers can be influenced by financial relationships while making judgments and decisions at a subconscious level such that they are not aware of the influence[31]. In the era of DAA treatment, the only published SRs providing the number of chronically HCV-infected individuals in the MENA region have substantial proportions of their authors who disclosed financial relationships with HCV DAA pharmaceutical companies. Our overview identifies a need in the MENA region for estimates of the number of chronically HCV-infected individuals who are candidates for DAA treatment by researchers who have not received financial assistance from Gilead Science.

Status of HCV epidemiology in the MENA region: Incidence
HCV infection is usually a slow progressive disease, which can lead to liver cirrhosis a couple of decades post-HCV infection[68]. Patients with liver cirrhosis are at 1%-5% annual risk of HCC and a 3%-6% annual risk of hepatic decompensation[68]. As such, a long period with non-specific symptoms passes before manifestations of HCV-related hepatic and extra-hepatic dysfunctions occur[69], which may then lead to the diagnosis of HCV infection. The distributions of these dysfunctions and diseases reflect past HCV infection transmission[69,70]. Hence, the prevalence of anti-HCV provides a retrospective snapshot of HCV transmission in a population, while the incidence describes the current HCV status in that population. Our overview also identifies research evidence gaps pertaining to up-to-date anti-HCV incidence data in all MENA countries. Therefore, we recommend that additional research be conducted to measure the HCV incidence to assess current HCV transmission trends. Incidence studies in the GP and populations at differing levels of risk for acquiring HCV will allow real-time measurement of HCV transmission. Nowcasting will help policymakers to develop and implement efficient prevention strategies alongside treatment programs.

Status of HCV epidemiology in the MENA region: Prevalence in the general population
Among the 20 MENA countries, anti-HCV prevalence in the GP varied widely. Egypt, Pakistan, and Yemen were classified as having high prevalences of anti-HCV. In the six Gulf Nations, namely, Bahrain, Kuwait, Oman, Qatar, Saudi Arabia, and the UAE, the reporting quality of the anti-HCV prevalence data was poor (no data for Bahrain). Nevertheless, the available data in the GP demonstrate low-to-moderate prevalences of anti-HCV in these countries. Remarkably, in these countries, the data from blood donors were of better reporting quality. Among blood donors, the anti-HCV prevalence was lower in nationals than in migrants, suggesting that the demographic characteristics of these Gulf Nations (nationals *vs* migrants) should be considered when describing the HCV epidemiology[71]. In these countries, population size increased rapidly in the last two decades due to the immigration of temporary workers[72]. More than 30% of the populations of the Gulf Nations are migrants; in Qatar and the UAE in 2014, this figure was over 80%[73,74]. In Qatar, Egyptian and Pakistani migrants accounted for 9% and 5% of the population, respectively, while Qatari nationals accounted for 12% (2016)[75]. HCV prevalence in a GP including both nationals and migrants in a country such as Qatar will be influenced by the HCV epidemiology profiles of the migrants' countries of origin. Of note, migrants have to be negative for HIV, HBV, and HCV upon arrival in the Gulf Nations; however, screening is not mandatory thereafter, except for some professions such as those in the healthcare sector[76-81]. Thus, HCV-infected migrants in these countries would most likely have been infected while visiting their countries of origin.

Status of HCV epidemiology in MENA: Prevalence in blood donors
In countries that (1) prohibit persons at higher risk of acquiring blood-borne infection from donating blood and (2) systematically implement pre-transfusion screening

of blood donation for these infections, the prevalence of blood-borne infections in blood donors should be lower than that in the GP[28,29]. In some MENA countries, the anti-HCV prevalence was higher in blood donors than in the GP. Furthermore, in MENA countries, prevalence and incidence measurements in transfused patients and in patients with blood disorders suggest that HCV transmission in healthcare settings is prevalent and is likely to be ongoing. There is a need to ensure that patients receive the safest possible blood products. The management of all blood units and their components should guarantee that only those with negative HCV screening assays are sent to the hospitals to be used in transfusions. Strong regulatory policies for all blood banks must be implemented and enforced to achieve this.

Status of HCV epidemiology in the MENA region: Prevalence in healthcare settings

In Pakistan, HCV transmission due to unsafe injections (*i.e.,* using contaminated needles and syringes) was identified in 2000[82], and in 2006, the proportion of people sharing injection equipment during their last injection was between 8.5% and 33.6%[83], suggesting that HCV transmission in healthcare settings was still ongoing. Additionally, we report that the prevalence of anti-HCV is higher in healthcare workers than in the GP in Iraq (2002-2010), Libya (1992-2004), Syria, and Yemen (study dates not reported). In 1999, HCV transmission in healthcare workers due to needle injuries from HCV-positive patients was reported in Pakistan[84]. The identified evidence suggests that occupational exposure to HCV occurs in healthcare settings in some MENA countries. In 2000, in order to prevent the transmission of HCV and other blood-borne infections in healthcare settings, the World Health Organization recommended the implementation of strategies targeting healthcare workers and patients, focusing on achieving behavioral changes to reduce re-use and over-use of injections, increase the exclusive use of sterile syringes and needles, and increase the proper disposal of sharp waste after use[85].

Status of HCV epidemiology in the MENA region: Key populations

Intravenous drug use appears to drive HCV infection in half the MENA countries, namely, Egypt, Lebanon, Libya, Morocco, Oman, Pakistan, Palestine, Saudi Arabia, Syria, and Tunisia. Relatively up-to-date data with good reporting quality were identified for these countries. However, missing data for the other MENA countries impact the evaluation of role of intravenous drug use in HCV transmission in a substantial part of the region. Prisoners are a key population rarely studied in MENA countries. We identified HCV outcomes in prisoners in only three countries (Libya, Iraq, and Lebanon). It appears that incarcerated individuals are at higher risk of acquiring HCV infections, especially

HIV positive prisoners. Similarly, HCV epidemiology in MSM was described only in Sudan and Lebanon, and no data from HIV-infected MSM were identified. Lebanon, Libya, Sudan, Syria, and Tunisia reported higher anti-HCV prevalences in female sex workers than in the GP, demonstrating that this population may be at higher risk of acquiring HCV infections. Consequently, population health research studies are needed in these key populations to comprehensively characterize HCV epidemiology in the MENA countries, which will facilitate better planning, development, and implementation of precise preventive strategies and treatment programs.

Status of HCV epidemiology in the MENA region: HBV and HIV co-infection, vertical transmission, and community

Co-infections with HCV and HBV or HIV, which share common modes of transmission, namely, through exposure to infected blood and sexual transmission[86], reminds us of the need for a holistic approach to tackle the population health issue of blood-borne infections. Additionally, the identified evidence regarding HCV morbidity (incidence and prevalence) in pregnant women and in children in Egypt, Pakistan, Saudi Arabia, and Yemen suggested that vertical HCV transmission may also occur. As for vertical transmission, little evidence was identified regarding community-acquired HCV infections. Additional research studies are needed to explore these transmission modes.

Status of HCV epidemiology in the MENA region: Genotype geographical pattern

Genotype/subtype 1b is omnipresent across the region. Genotype 4 appears to be the most common in Egypt. In the Gulf Nations, the dominance of genotype 4 might be explained by the high proportion of migrants from Egypt. Genotype 3 is the most common in Pakistan, suggesting that intravenous drug use is likely the driver of HCV transmission in this country[87,88]. Genotype 3a in Pakistan appears to have emerged around the 1920s[88].

Many SRs reported HCV genotype/subtype distributions and pooled frequencies without specifying the temporality of their identified geographical pattern. HCV genotypes differ according to the mode of transmission. For instance, while HCV-1b is the most prevalent HCV genotype/subtype globally[6], HCV-3 and 1a are linked to PWID, and in Asia, HCV-1a is also linked to PWID[87]. A birth cohort analysis demonstrated that the distribution of HCV genotypes is changing in Japan and appears to be associated with the establishment of the prevention of HCV transmission through blood transfusions or nosocomial infections in 1990[89]. In Japan, genotype 1b is associated with the former mode of transmission, while genotypes 2a and 2b are associated with the latter mode of transmission[89]. Unfortunately, because of how genotype/subtype data are pooled and reported for the MENA region, we were not able to identify differences in time trends and geographic patterns.

Quality assessment, strengths and limitations of our overview

We followed PRISMA guidelines[11-13] in reporting our work. All PRISMA required items regarding the abstract and the different sections of the main text were reported. Furthermore, we assessed our methodology using the AMSTAR tool[14]. We answered eight of the 11 questions of this tool in the affirmative and reported the pages where the information can be found. The three remaining questions were not applicable to our work (*e.g.,* "Were the methods used to combine the findings of studies appropriate[14]? Was not applicable because we did not conduct a meta-analysis).

The quality assessment of SRs we conducted was limited to all SRs of HCV epidemiology in the MENA region that were published in peer-reviewed journals indexed on Medline as identified through PubMed, which may not be representative of SRs of HCV epidemiology in the MENA region in general. However, PubMed has been recognized as the first public digital archive; as such, we can assume that we have assessed the quality of the majority of SRs of HCV epidemiology published in peer-reviewed journals[90]. Additionally, we critically synthesized all available data on HCV morbidity and risk factors reported by those SRs. Knowing that most of the 37 SRs included in our overviews conducted a multi-source literature search including, in some SRs, a search for gray literature, we assumed that our synthesis in this overview is comprehensive. However, we might have missed some studies published recently that were not included in the SRs included in our overview. For instance, in Egypt, anti-HCV prevalence was estimated to be 6% in 2014[5], yet none of the SRs we included reported that estimate.

CONCLUSION

To reach the 2030 HCV infection elimination goal set by the GHSS-VH[67], evidence-based health policies need to be developed and implemented to optimize the allocation of financial resources and to alleviate the burden of disease due to HCV infection. Correct measures of anti-HCV prevalence, viremia, and genotype distribution are essential for developing strategies to manage and eliminate HCV infection. Evidence gap mapping and quality assessment, along with reviewing reported conflicts of interest, revealed countries and specific populations where data are scarce and/or lacking in quality. We identified areas of opportunity to further develop population health research to comprehensively characterize HCV infection in the MENA region. Currently, the available estimates of the numbers of chronically HCV-infected patients who are candidates for the new DAA regimens are computed by researchers, a substantial proportion of whom have indirect or direct financial relationships with HCV DAA pharmaceutical companies. Additionally, a substantial proportion of the included SRs did not report precise outcomes, which can lead to misinterpretation of the data. Evidence-based policies should be established from up-to-date information generated from rigorously designed studies and reported outcomes.

Our synthesis of the published SRs of HCV epidemiology in the MENA region demonstrates that despite the region being recognized as having the greatest HCV burden, a majority of the 20 countries have low to moderate anti-HCV prevalence. Only Egypt, Pakistan, and Yemen demonstrated a high anti-HCV prevalence. Iatrogenic transmission of HCV in patients and occupational exposure in healthcare workers are likely to be ongoing in the MENA countries. There is an urgent need for safer blood products and the implementation of safe medical practice guidelines. Intravenous drug use appears to be another common mode of HCV transmission in half the MENA countries. Research studies are needed to characterize the status of HCV epidemiology in PWID in countries where data are lacking. To develop effective prevention strategies and treatment programs to achieve HCV elimination, characterizing HCV epidemiology in key populations such as MSM and sex workers is essential and cannot be overlooked.

ARTICLE HIGHLIGHTS

Research background

The Middle East and North Africa (MENA) has been identified as the region most affected by hepatitis C virus (HCV) infection worldwide predominantly due to the high HCV infection burden in Egypt and Pakistan. Since 2013, chronic infections with HCV genotypes 1, 2, 3, and 4 are curable, making elimination of HCV infection achievable for the first time. However, access and availability of the new direct-acting antiviral drug (DAA) regimens remains a challenge owing to country-level economic matters.

Research motivation

Developing and prioritizing evidence-based strategies for prevention programs and treatment scale-up require up-to-date information generated from rigorously designed studies and reported outcomes. A substantial number of systematic reviews of HCV infection in the MENA region have been published. What is the quality of these systematic reviews and what is the quality of the studies included in these systematic reviews? Researchers can be influenced by financial relationships while making judgments and decisions at a subconscious level such that they are not aware of the influence. The use and interpretation of the outcomes in the systematic reviews need to be viewed keeping in mind the reported conflict of interests by the authors of the systematic reviews. Our overview was also motivated because of the following questions: what do we know about hepatitis C epidemiology in the MENA region? High country-level anti-HCV prevalence has been identified in Egypt and Pakistan. What is the status of the other 18 MENA countries? What are the identified potential modes of transmission and populations at higher risk of acquiring hepatitis C infection?

Research objectives

The primary objective of our overview is to assess the quality of the data reported by the published systematic reviews of HCV epidemiology in the MENA countries taking into account conflict of interest disclosed by the authors of these systematic reviews. Our secondary objective is to produce a comprehensive picture of HCV infection epidemiology in the 20 countries of the MENA region.

Research methods

An *a priori* protocol of our overview is registered with the International Prospective Register of Systematic Reviews (PROSPERO registration number CRD42017076736). We conducted an overview of systematic reviews based

on the Cochrane Handbook for Systematic Reviews of Interventions. We used broad search criteria to include all systematic reviews on HCV infection in the MENA region published after 2008 - the publication year of the first version of the Cochrane Handbook for Systematic Reviews of Interventions. We extracted all relevant outcomes related to HCV infection epidemiology. The nine primary outcomes of interest were HCV antibody (anti-) prevalences and incidences in different at-risk populations; the HCV viremic (RNA positive) rate in HCV-positive individuals; HCV viremic prevalence in the general population; the prevalence of HCV co-infection with the hepatitis B virus, human immunodeficiency virus, or schistosomiasis; the HCV genotype/subtype distribution; and the risk factors for HCV transmission. Thereafter, we critically analyzed and synthesized the extracted data by assessing their quality - using PICOTS framework, by evaluating conflict of interests disclosed by the authors of the systematic reviews, and by mapping the evidence and the gaps. Our overview is reported following PRISMA guidelines and assessed using AMSTAR tool.

Research results

Data reporting in a substantial proportion of systematic reviews on HCV infection in MENA may mislead the interpretation of these data. Our overview identified that a substantial proportion of the systematic reviews failed to report their outcomes following the PICOTS framework. Additionally, a substantial proportion of the systematic reviews lacked the key identified features of transparency and pragmatism. Our overview emphasizes the significance of considering the disclosure of financial relationships while assessing research and its reported outcomes. In the era of DAA treatment, the only published systematic reviews providing the number of chronically HCV-infected individuals in the MENA region - number of candidates for DAA treatment, have a large number of authors who disclosed financial relationships with HCV DAA pharmaceutical companies. We identified low prevalence of anti-HCV (< 1.5%) in Djibouti, Kuwait, Oman, Qatar, and UAE; moderate prevalence (1.5%-3.5%) in Algeria, Iraq, Lebanon, Libya, Morocco, Saudi Arabia, Sudan, Syria, and Tunisia; and high prevalence (> 3.5%) in Egypt, Pakistan, and Yemen. In Pakistan, anti-HCV prevalence may have increased from 5.4% in 1996-2007 to 6.7% in 2007-2008. No reported data in the general population was identified for Bahrain, Jordan, or Palestine. Intravenous drug use appears to drive HCV infection in Egypt, Lebanon, Libya, Morocco, Oman, Pakistan, Palestine, Saudi Arabia, Syria, and Tunisia. Relatively up-to-date data with good reporting quality were identified for these countries. However, missing data for the other ten MENA countries affect the evaluation of role of intravenous drug use in HCV transmission in a substantial part of the region. Furthermore, we identified that HCV transmission most likely occurs in healthcare settings due to iatrogenic and/or occupational exposures. Community-acquired HCV infection may also occur in the MENA countries; however, we identified evidence gaps in the region. Often, anti-HCV prevalence measures were not identified for key populations such as prisoners, men who have sex with men, and sex workers. Anti-HCV prevalence data were predominantly missing for the countries of the Gulf Cooperation Council and Yemen.

Research conclusions

Correct measures of anti-HCV prevalence, viremia, and genotype distribution are essential for developing strategies to manage and eliminate HCV infection. Evidence gap mapping and quality assessment, along with reviewing reported authors conflict of interest, revealed MENA countries and specific populations where data are scarce and/or lacking in quality. Our overview comprehensively characterizes hepatitis C epidemiology in the 20 countries of the MENA region and emphasizes their needs in terms of treatment and prevention. This will help policy makers of these countries to develop and prioritize evidence-based strategies for prevention programs and treatment scale-up in the region. This overview will contribute to the improvement of population health research practices in order to build research capacity in the MENA region

Research perspectives

Future systematic reviews on HCV epidemiology in the MENA region should strive to provide accurate information on HCV epidemiology demonstrating practical and clinical significance and relevance. To facilitate the development of precise prevention strategies and treatment programs, we recommend when estimating and reporting HCV prevalence and incidence, to avoid mixing populations at differing risk of acquiring HCV. Furthermore, up-to-date and good

quality data are required in order to nowcast HCV epidemiology in the region. When data is available for long time periods, reporting should reflect changes in prevalence or incidence over time, which will allow to accurately describe the past and current HCV epidemiology in a population.

REFERENCES

1   **World Health Organization**. Hepatitis C Geneva, Switzerland 2017 [cited 2017-09-27] Available from: URL: http://www.who.int/mediacentre/factsheets/fs164/en/

2   **World Health Organization**. Hepatitis B Geneva, Switzerland 2017[cited 2017-09-27] Available from: URL: http://www.who.int/mediacentre/factsheets/fs204/en/

3   **Frank C**, Mohamed MK, Strickland GT, Lavanchy D, Arthur RR, Magder LS, El Khoby T, Abdel-Wahab Y, Aly Ohn ES, Anwar W, Sallam I. The role of parenteral antischistosomal therapy in the spread of hepatitis C virus in Egypt. *Lancet* 2000; **355**: 887-891 [PMID: 10752705 DOI: 10.1016/S0140-6736(99)06527-7]

4   **Al-Zanaty F**, Way A. Egypt Demographic and Health Survey 2008. Cairo, Egypt: Ministry of Health, 2009

5   **Al-Zanaty F**. Egypt Demographic and Health Survey 2014. Cairo, Egypt: Ministry of Health and Population 2015

6   **Gower E**, Estes C, Blach S, Razavi-Shearer K, Razavi H. Global epidemiology and genotype distribution of the hepatitis C virus infection. *J Hepatol* 2014; **61**: S45-S57 [PMID: 25086286 DOI: 10.1016/j.jhep.2014.07.027]

7   **Higgins J**, Green S. Cochrane Handbook for Systematic Reviews of Interventions Version 5.1.0 The Cochrane Collaboration; 2011

8   **Ioannidis JP**, Fanelli D, Dunne DD, Goodman SN. Meta-research: Evaluation and Improvement of Research Methods and Practices. *PLoS Biol* 2015; **13**: e1002264 [PMID: 26431313 DOI: 10.1371/journal.pbio.1002264]

9   **Chaabna K**, Cheema S, Abraham A, Alrouh H, Mamtani R, Sheikh JI. Gray literature in Systematic Reviews on Population Health in the Middle East and North Africa: Protocol of an Overview of Systematic Reviews and Evidence Mapping. *Syst Rev* 2018 [DOI: 10.1186/s13643-018-0751-4]

10   **Booth A**, Clarke M, Dooley G, Ghersi D, Moher D, Petticrew M, Stewart L. PROSPERO at one year: an evaluation of its utility. *Syst Rev* 2013; **2**: 4 [PMID: 23320413 DOI: 10.1186/2046-4053-2-4]

11   **Moher D**, Liberati A, Tetzlaff J, Altman DG; PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *J Clin Epidemiol* 2009; **62**: 1006-1012 [PMID: 19631508 DOI: 10.1016/j.jclinepi.2009.06.005]

12   **Liberati A**, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JP, Clarke M, Devereaux PJ, Kleijnen J, Moher D. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. *J Clin Epidemiol* 2009; **62**: e1-e34 [PMID: 19631507 DOI: 10.1016/j.jclinepi.2009.06.006]

13   **Beller EM**, Glasziou PP, Altman DG, Hopewell S, Bastian H, Chalmers I, Gøtzsche PC, Lasserson T, Tovey D; PRISMA for Abstracts Group. PRISMA for Abstracts: reporting systematic reviews in journal and conference abstracts. *PLoS Med* 2013; **10**: e1001419 [PMID: 23585737 DOI: 10.1371/journal.pmed.1001419]

14   **Shea BJ**, Grimshaw JM, Wells GA, Boers M, Andersson N, Hamel C, Porter AC, Tugwell P, Moher D, Bouter LM. Development of AMSTAR: a measurement tool to assess the methodological quality of systematic reviews. *BMC Med Res Methodol* 2007; **7**: 10 [PMID: 17302989 DOI: 10.1186/1471-2288-7-10]

15   **United Nations**. Total Population - Both Sexes. De facto population in a country, area or region as of 1 July of the year indicated.

Figures are presented in thousands. World Population Prospects: The 2015 Revision 2016 [cited 2017-09-10] Available from: URL: https://esa.un.org/unpd/wpp/Download/Standard/Population/

16   **National institutes of Health**. PubMed.gov US National Library of Medcine Bethesda, USA2016 [cited 2017-09-10] Available from: URL: http://www.ncbi.nlm.nih.gov/pubmed

17   Clarivate Analytics. EndNote X7 2017. [cited 2017-09-10]Available from: URL: http://endnote.com/product-details

18   **Elmagarmid A**, Fedorowicz Z, Hammady H, Ilyas I, Khabsa M, Ouzzani M, editors. Rayyan: a systematic reviews web app for exploring and filtering searches for eligible studies for Cochrane Reviews. Evidence-Informed Public Health: Opportunities and Challenges Abstracts of the 22nd Cochrane Colloquium, 2014; Hyderabad, India

19   **Ouzzani M**, Hammady H, Fedorowicz Z, Elmagarmid A. Rayyan-a web and mobile app for systematic reviews. *Syst Rev* 2016; **5**: 210 [PMID: 27919275 DOI: 10.1186/s13643-016-0384-4]

20   **Gilead Science.** U.S. Food and Drug Administration Approves Gilead's Vosevi™ (Sofosbuvir/Velpatasvir/Voxilaprevir) for Re-Treatment of Adults with Chronic Hepatitis C Virus: Gilead Sciences, Inc.; 2017 [cited 2017-12-10]. Available from: URL: http://www.gilead.com/news/press-releases/2017/7/us-food-and-drug-administration-approves-gileads-vosevi-sofosbuvirvelpatasvirvoxilaprevir-for-retreatment-of-adults-with-chronic-hepatitis-c-virus

21   **Sulkowski MS**, Gardiner DF, Rodriguez-Torres M, Reddy KR, Hassanein T, Jacobson I, Lawitz E, Lok AS, Hinestrosa F, Thuluvath PJ, Schwartz H, Nelson DR, Everson GT, Eley T, Wind-Rotolo M, Huang SP, Gao M, Hernandez D, McPhee F, Sherman D, Hindes R, Symonds W, Pasquinelli C, Grasela DM; AI444040 Study Group. Daclatasvir plus sofosbuvir for previously treated or untreated chronic HCV infection. *N Engl J Med* 2014; **370**: 211-221 [PMID: 24428467 DOI: 10.1056/NEJMoa1306218]

22   **Rouabhia S**, Baghazza S, Sadouki H, Djezzar S, Bencherif R, Guehimeche R, Sadelaoud M, Mallem D, Chaabna K. Ventricular Extrasystoles after First Dose of Sofosbuvir in a Patient Treated with Propranolol but not with Amiodarone: A Case Report. *Rev Recent Clin Trials* 2017; **12**: 159-161 [PMID: 28745218 DOI: 10.2174/1574887112666170725132044]

23   **Fontaine H**, Lazarus A, Pol S, Pecriaux C, Bagate F, Sultanik P, Boueyre E, Corouge M, Mallet V, Vallet-Pichard A, Sogni P, Duboc D; Cochin Hepatology and Cardiology Group. Bradyarrhythmias Associated with Sofosbuvir Treatment. *N Engl J Med* 2015; **373**: 1886-1888 [PMID: 26535533 DOI: 10.1056/NEJMc1505967]

24   **Dutt A**. Will Gilead & AbbVie Benefit from Merck's Decision to Drop Two HCV Programs? : Nasdaq; 2017 [cited 2018-03-21] Available from: URL: https://www.nasdaq.com/article/will-gilead-abbvie-benefit-from-mercks-decision-to-drop-two-hcv-programs-cm854557

25   **PubMed Health**. PICO Framework Bethesda MD, USA: U.S. National Library of Medicine; 2017 [cited 2018-02-06] Available from: URL: https://www.ncbi.nlm.nih.gov/pubmedhealth/PMHT0029906/

26   **Thompson M**, Tiwari A, Fu R. Results. Existing Literature on Improving the Use of Systematic Reviews To Inform Study Designs. A Framework to faciliatate the use of systematic reviews and meta-analyses in the design of primary research studies [Internet]. Rockville (MD), US: Agency for Healthcare Research and Quality; 2012

27   **Chaabna K**, Kouyoumjian SP, Abu-Raddad LJ. Hepatitis C Virus Epidemiology in Djibouti, Somalia, Sudan, and Yemen: Systematic Review and Meta-Analysis. *PLoS One* 2016; **11**: e0149966 [PMID: 26900839 DOI: 10.1371/journal.pone.0149966]

28   **Denniston MM**, Jiles RB, Drobeniuc J, Klevens RM, Ward JW, McQuillan GM, Holmberg SD. Chronic hepatitis C virus infection in the United States, National Health and Nutrition Examination Survey 2003 to 2010. *Ann Intern Med* 2014; **160**: 293-300 [PMID: 24737271 DOI: 10.7326/M13-1133]

29   **Zou S**, Dorsey KA, Notari EP, Foster GA, Krysztof DE, Musavi F, Dodd RY, Stramer SL. Prevalence, incidence, and residual risk of human immunodeficiency virus and hepatitis C virus infections among United States blood donors since the introduction of nucleic acid testing. *Transfusion* 2010; **50**: 1495-1504 [PMID: 20345570 DOI: 10.1111/j.1537-2995.2010.02622.x]

30   **Mohd Hanafiah K**, Groeger J, Flaxman AD, Wiersma ST. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. *Hepatology* 2013; **57**: 1333-1342 [PMID: 23172780 DOI: 10.1002/hep.26141]

31   **Resnik DB**, Elliott KC. Taking financial relationships into account when assessing research. *Account Res* 2013; **20**: 184-205 [PMID: 23672544 DOI: 10.1080/08989621.2013.788383]

32   **Alavian SM**, Aalaei-Andabili SH. Lack of Knowledge About Hepatitis C Infection Rates Among Patients With Inherited Coagulation Disorders in Countries Under the Eastern Mediterranean Region Office of WHO (EMRO): A Meta-Analysis. *Hepat Mon* 2012; **12**: 244-252 [PMID: 22690231 DOI: 10.5812/hepatmon.6050]

33   **Chemaitelly H**, Chaabna K, Abu-Raddad LJ. The Epidemiology of Hepatitis C Virus in the Fertile Crescent: Systematic Review and Meta-Analysis. *PLoS One* 2015; **10**: e0135281 [PMID: 26296200 DOI: 10.1371/journal.pone.0135281]

34   **Nelson PK**, Mathers BM, Cowie B, Hagan H, Des Jarlais D, Horyniak D, Degenhardt L. Global epidemiology of hepatitis B and hepatitis C in people who inject drugs: results of systematic reviews. *Lancet* 2011; **378**: 571-583 [PMID: 21802134 DOI: 10.1016/S0140-6736(11)61097-0]

35   **Riou J**, Aït Ahmed M, Blake A, Vozlinsky S, Brichler S, Eholié S, Boëlle PY, Fontanet A; HCV epidemiology in Africa group. Hepatitis C virus seroprevalence in adults in Africa: a systematic review and meta-analysis. *J Viral Hepat* 2016; **23**: 244-255 [PMID: 26477881 DOI: 10.1111/jvh.12481]

36   **Alavian SM**, Haghbin H. Relative Importance of Hepatitis B and C Viruses in Hepatocellular Carcinoma in EMRO Countries and the Middle East: A Systematic Review. *Hepat Mon* 2016; **16**: e35106 [PMID: 27226803 DOI: 10.5812/hepatmon.35106]

37   **Ghaderi-Zefrehi H**, Gholami-Fesharaki M, Sharafi H, Sadeghi F, Alavian SM. The Distribution of Hepatitis C Virus Genotypes in Middle Eastern Countries: A Systematic Review and Meta-Analysis. *Hepat Mon* 2016; **16**: e40357 [PMID: 27826320 DOI: 10.5812/hepatmon.40357]

38   **Ramia S**, Melhem NM, Kreidieh K. Hepatitis C virus infection in the Middle East and North Africa "MENA" region: injecting drug users (IDUs) is an under-investigated population. *Infection* 2012; **40**: 1-10 [PMID: 22237470 DOI: 10.1007/s15010-011-0236-z]

39   **El-Ghitany EM**, Abdel Wahab MM, Abd El-Wahab EW, Hassouna S, Farghaly AG. A comprehensive hepatitis C virus risk factors meta-analysis (1989-2013): do they differ in Egypt? *Liver Int* 2015; **35**: 489-501 [PMID: 24923487 DOI: 10.1111/liv.12617]

40   **Waheed Y**, Shafi T, Safi SZ, Qadri I. Hepatitis C virus in Pakistan: a systematic review of prevalence, genotypes and risk factors. *World J Gastroenterol* 2009; **15**: 5647-5653 [PMID: 19960560 DOI: 10.3748/wjg.15.5647]

41   **Umar M**, Khaar HT, Khurram M, Hasan Z. Anti-HCV antibody positivity of various sections of Pakistani patients. *J Coll Physicians Surg Pak* 2009; **19**: 737-741 [PMID: 19889276]

42   **Ezzikouri S**, Pineau P, Benjelloun S. Hepatitis C virus infection in the Maghreb region. *J Med Virol* 2013; **85**: 1542-1549 [PMID: 23780703 DOI: 10.1002/jmv.23643]

43   **Karoney MJ**, Siika AM. Hepatitis C virus (HCV) infection in Africa: a review. *Pan Afr Med J* 2013; **14**: 44 [PMID: 23560127 DOI: 10.11604/pamj.2013.14.44.2199]

44   **Gasim GI**, Murad IA, Adam I. Hepatitis B and C virus infections among pregnant women in Arab and African countries. *J Infect Dev Ctries* 2013; **7**: 566-578 [PMID: 23949291 DOI: 10.3855/jidc.3243]

45   **Van-Lume DS**, Albuquerque Mde F, Souza AI, Domingues AL, Lopes EP, Morais CN, Montenegro SM. Association between Schistosomiasis mansoni and hepatitis C: systematic review. *Rev Saude Publica* 2013; **47**: 414-424 [PMID: 24037369 DOI: 10.1590/S0034-910.2013047004247]

46   **Gasim GI**, Bella A, Adam I. Schistosomiasis, hepatitis B and hepatitis C co-infection. *Virol J* 2015; **12**: 19 [PMID: 25889398

DOI: 10.1186/s12985-015-0251-2]

47   **de Martel C**, Maucort-Boulch D, Plummer M, Franceschi S. World-wide relative contribution of hepatitis B and C viruses in hepatocellular carcinoma. *Hepatology* 2015; **62**: 1190-1200 [PMID: 26146815 DOI: 10.1002/hep.27969]

48   **Hussein WM**, Anwar WA, Attaleb M, Mazini L, Försti A, Trimbitas RD, Khyatti M. A review of the infection-associated cancers in North African countries. *Infect Agent Cancer* 2016; **11**: 35 [PMID: 27512409 DOI: 10.1186/s13027-016-0083-8]

49   **Azevedo TC**, Zwahlen M, Rauch A, Egger M, Wandeler G. Hepatitis C in HIV-infected individuals: a systematic review and meta-analysis of estimated prevalence in Africa. *J Int AIDS Soc* 2016; **19**: 20711 [PMID: 27293220 DOI: 10.7448/IAS.19.1.20711]

50   **Sievert W**, Altraif I, Razavi HA, Abdo A, Ahmed EA, Alomair A, Amarapurkar D, Chen CH, Dou X, El Khayat H, Elshazly M, Esmat G, Guan R, Han KH, Koike K, Largen A, McCaughan G, Mogawer S, Monis A, Nawaz A, Piratvisuth T, Sanai FM, Sharara AI, Sibbel S, Sood A, Suh DJ, Wallace C, Young K, Negro F. A systematic review of hepatitis C virus epidemiology in Asia, Australia and Egypt. *Liver Int* 2011; **31 Suppl 2**: 61-80 [PMID: 21651703 DOI: 10.1111/j.1478-3231.2011.02540.x]

51   **Bahakim H**, Bakir TM, Arif M, Ramia S. Hepatitis C virus antibodies in high-risk Saudi groups. *Vox Sang* 1991; **60**: 162-164 [PMID: 1907415 DOI: 10.1111/j.1423-0410.1991.tb00894.x]

52   **Fadlalla FA**, Mohamoud YA, Mumtaz GR, Abu-Raddad LJ. The epidemiology of hepatitis C virus in the Maghreb region: systematic review and meta-analyses. *PLoS One* 2015; **10**: e0121873 [PMID: 25803848 DOI: 10.1371/journal.pone.0121873]

53   **Bosan A**, Qureshi H, Bile KM, Ahmad I, Hafiz R. A review of hepatitis viral infections in Pakistan. *J Pak Med Assoc* 2010; **60**: 1045-1058 [PMID: 21381562]

54   **Abozaid SM**, Shoukri M, Al-Qahtani A, Al-Ahdal MN. Prevailing genotypes of hepatitis C virus in Saudi Arabia: a systematic analysis of evidence. *Ann Saudi Med* 2013; **33**: 1-5 [PMID: 23458931 DOI: 10.5144/0256-4947.2013.1]

55   **Attaullah S**, Khan S, Ali I. Hepatitis C virus genotypes in Pakistan: a systemic review. *Virol J* 2011; **8**: 433 [PMID: 21902822 DOI: 10.1186/1743-422X-8-433]

56   **Bruggmann P**, Berg T, Øvrehus AL, Moreno C, Brandão Mello CE, Roudot-Thoraval F, Marinho RT, Sherman M, Ryder SD, Sperl J, Akarca U, Balık I, Bihl F, Bilodeau M, Blasco AJ, Buti M, Calinas F, Calleja JL, Cheinquer H, Christensen PB, Clausen M, Coelho HS, Cornberg M, Cramp ME, Dore GJ, Doss W, Duberg AS, El-Sayed MH, Ergör G, Esmat G, Estes C, Falconer K, Félix J, Ferraz ML, Ferreira PR, Frankova S, García-Samaniego J, Gerstoft J, Giria JA, Gonçales FL Jr, Gower E, Gschwantler M, Guimarães Pessôa M, Hézode C, Hofer H, Husa P, Idilman R, Kåberg M, Kaita KD, Kautz A, Kaymakoglu S, Krajden M, Krarup H, Laleman W, Lavanchy D, Lázaro P, Marotta P, Mauss S, Mendes Correa MC, Müllhaupt B, Myers RP, Negro F, Nemecek V, Örmeci N, Parkes J, Peltekian KM, Ramji A, Razavi H, Reis N, Roberts SK, Rosenberg WM, Sarmento-Castro R, Sarrazin C, Semela D, Shiha GE, Sievert W, Stärkel P, Stauber RE, Thompson AJ, Urbanek P, van Thiel I, Van Vlierberghe H, Vandijck D, Vogel W, Waked I, Wedemeyer H, Weis N, Wiegand J, Yosry A, Zekry A, Van Damme P, Aleman S, Hindman SJ. Historical epidemiology of hepatitis C virus (HCV) in selected countries. *J Viral Hepat* 2014; **21 Suppl 1**: 5-33 [PMID: 24713004 DOI: 10.1111/jvh.12247]

57   **Raja NS**, Janjua KA. Epidemiology of hepatitis C virus infection in Pakistan. *J Microbiol Immunol Infect* 2008; **41**: 4-8 [PMID: 18327420]

58   **Sadeghi F**, Salehi-Vaziri M, Almasi-Hashiani A, Gholami-Fesharaki M, Pakzad R, Alavian SM. Prevalence of Hepatitis C Virus Genotypes Among Patients in Countries of the Eastern Mediterranean Regional Office of WHO (EMRO): A Systematic Review and Meta-Analysis. *Hepat Mon* 2016; **16**: e35558 [PMID: 27274353 DOI: 10.5812/hepatmon.35558]

59   **Mohamoud YA**, Riome S, Abu-Raddad LJ. Epidemiology of hepatitis C virus in the Arabian Gulf countries: Systematic review and meta-analysis of prevalence. *Int J Infect Dis* 2016; **46**: 116-125

[PMID: 26996460 DOI: 10.1016/j.ijid.2016.03.012]

60   **Reker C**, Islam KM. Risk factors associated with high prevalence rates of hepatitis C infection in Egypt. *Int J Infect Dis* 2014; **25**: 104-106 [PMID: 24865321 DOI: 10.1016/j.ijid.2014.02.003]

61   **Mohamoud YA**, Mumtaz GR, Riome S, Miller D, Abu-Raddad LJ. The epidemiology of hepatitis C virus in Egypt: a systematic review and data synthesis. *BMC Infect Dis* 2013; **13**: 288 [PMID: 23799878 DOI: 10.1186/1471-2334-13-288]

62   **Ali SA**, Donahue RM, Qureshi H, Vermund SH. Hepatitis B and hepatitis C in Pakistan: prevalence and risk factors. *Int J Infect Dis* 2009; **13**: 9-19 [PMID: 18835208 DOI: 10.1016/j.ijid.2008.06.019]

63   **Bajubair MA**, Elrub AA, Bather G. Hepatic viral infections in Yemen between 2000--2005. *Saudi Med J* 2008; **29**: 871-874 [PMID: 18521468]

64   **World Health Organization.** Global Hepatitis Report 2017. Geneva, Switzerland: World Health Organization, 2017

65   **Lehman EM**, Wilson ML. Epidemiology of hepatitis viruses among hepatocellular carcinoma cases and healthy people in Egypt: a systematic review and meta-analysis. *Int J Cancer* 2009; **124**: 690-697 [PMID: 18973270 DOI: 10.1002/ijc.23937]

66   **Ioannidis JP**. Why Most Clinical Research Is Not Useful. *PLoS Med* 2016; **13**: e1002049 [PMID: 27328301 DOI: 10.1371/journal.pmed.1002049]

67   **Hutin YJ**, Wiktor SZ. Prioritising treatment among people who inject drugs in order to eliminate hepatitis C: addressing reluctance with sound economic analyses. *Gut* 2017; **66**: 2052-2053 [PMID: 27566128 DOI: 10.1136/gutjnl-2016-312636]

68   **Westbrook RH**, Dusheiko G. Natural history of hepatitis C. *J Hepatol* 2014; **61**: S58-S68 [PMID: 25443346 DOI: 10.1016/j.jhep.2014.07.012]

69   **Chaabna K**, Boniol M, de Vuyst H, Vanhems P, Antônio de Ávila Vitoria M, Curado MP. Geographical patterns of Kaposi's sarcoma, nonHodgkin lymphomas, and cervical cancer associated with HIV infection in five African populations. *Eur J Cancer Prev* 2012; **21**: 1-9 [PMID: 21955799 DOI: 10.1097/CEJ.0b013e32834a802a]

70   **Chaabna K**, Soerjomataram I, Rouabhia S, Chichoune S, Scholtes C, Vanhems P, Saidi M, Forman D. Co-occurrence of diffuse large B cell non-hodgkin lymphoma and chronic hepatitis C in Algerian patients: two case reports. *J Cancer Res Ther* 2013; **9**: 532-533 [PMID: 24126002 DOI: 10.4103/0973-1482.119372]

71   **Chaabna K**, Cheema S, Mamtani R. Migrants, healthy worker effect, and mortality trends in the Gulf Cooperation Council countries. *PLoS One* 2017; **12**: e0179711 [PMID: 28632794 DOI: 10.1371/journal.pone.0179711]

72   **Abdul Salam A**, Elsegaey I, Khraif R, AlMutairi A, Aldosari A. Components and public health impact of population growth in the Arab world. *PLoS One* 2015; **10**: e0124944 [PMID: 25993053 DOI: 10.1371/journal.pone.0124944]

73   **De Bel-Air F**. Gulf Labour Markets and Migration (GLMM). Demography, migration and labour market in Qatar. GLMM - EN - No. 8/2014. San Domenico di Fiesole, Italy: European University Institute and Gulf Research Center (GRC), 2015

74   **De Bel-Air F.** Gulf Labour Markets and Migration (GLMM). Demography, Migration, and the Labour Market in the UAE. GLMM - EN - No. 7/2015. San Domenico di Fiesole, Italy: European University Institute and Gulf Research Center (GRC), 2015

75   **Priya DSouza Consultancy.** Population of Qatar by nationality - 2017 report. Doha, Qatar: 2017

76   **Kuwait Visa**. Visa types 2017 [cited 2017-05-21] Available from: URL: http://kuwaitvisa.com/visa-types/

77   **Labour Market Regulatory Authority**. Pre-employment Examination General Rules for Expatriates Kingdom of Bahrain N.D. [cited 2017-01-11] Available from: URL: http://www.ilo.org/wcmsp5/groups/public/---ed_protect/---protrav/---ilo_aids/documents/legaldocument/wcms_241688.pdf

78   **Mininstry of Public Health**. Medical commission Doha, Qatar 2017 [cited 2017-01-11]. Available from: URL: https://www.moph.gov.qa/health-services/services-to-public/medical-commission/medical-commission

79 **Health Authority**. Residence-Visa Medical Check-up Abu Dhabi, United Arab Emirates2017 [cited 2017-01-11]. Available from: URL: https://www.haad.ae/haad/tabid/1200/Default.aspx

80 **Royal Oman Police - Directorate General of Operations**. Employment Visa 2017 [cited 2017-05-21]. Available from: URL: http://www.rop.gov.om/old/english/dg_pr_visas_employment.asp

81 **Embassy of the Kingdom of Saudi Arabia in United Kingdom**. Requirement for work visa 2017 [cited 2017-05-21] Available from: URL: http://embassies.mofa.gov.sa/sites/uk/EN/DiplomaticMission Services/Consulates/ConsulatesServices/Other%20Visas%20Servic es%20e/Pages/REQUIREMENTS%20FOR%20Work12%20VISA S.aspx

82 **Khan AJ**, Luby SP, Fikree F, Karim A, Obaid S, Dellawala S, Mirza S, Malik T, Fisher-Hoch S, McCormick JB. Unsafe injections and the transmission of hepatitis B and C in a periurban community in Pakistan. *Bull World Health Organ* 2000; **78**: 956-963 [PMID: 10994278]

83 **Altaf A**, Saleem N, Abbas S, Muzaffar R. High prevalence of HIV infection among injection drug users (IDUs) in Hyderabad and Sukkur, Pakistan. *J Pak Med Assoc* 2009; **59**: 136-140 [PMID: 19288936]

84 **Hamid SS**, Farooqui B, Rizvi Q, Sultana T, Siddiqui AA. Risk of transmission and features of hepatitis C after needlestick injuries. *Infect Control Hosp Epidemiol* 1999; **20**: 63-64 [PMID: 9927271 DOI: 10.1086/501547]

85 **World Health Organization**. Unsafe Injection Practices Having Serious Large-Scale Consequences. Press Release WHO/14. Geneva, Switzerland: World Health Organization, 2000

86 **World Health Organization**. What is hepatitis? 2016 [cited 2018-03-21]. Available from: URL: http://www.who.int/features/qa/76/en/

87 **Robaeys G**, Bielen R, Azar DG, Razavi H, Nevens F. Global genotype distribution of hepatitis C viral infection among people who inject drugs. *J Hepatol* 2016; **65**: 1094-1103 [PMID: 27520879 DOI: 10.1016/j.jhep.2016.07.042]

88 **Omata M**, Kanda T, Yokosuka O, Crawford D, Al-Mahtab M, Wei L, Ibrahim A, Lau GK, Sharma BC, Hamid SS, Chuang WL, Dokmeci AK. Features of hepatitis C virus infection, current therapies and ongoing clinical trials in ten Asian Pacific countries. *Hepatol Int* 2015; **9**: 486-507 [PMID: 25941137 DOI: 10.1007/s12072-015-9630-4]

89 **Toyoda H**, Kumada T, Takaguchi K, Shimada N, Tanaka J. Changes in hepatitis C virus genotype distribution in Japan. *Epidemiol Infect* 2014; **142**: 2624-2628 [PMID: 24598252 DOI: 10.1017/S0950268814000478]

90 **Masic I**, Milinovic K. On-line biomedical databases-the best source for quick search of the scientific information in the biomedicine. *Acta Inform Med* 2012; **20**: 72-84 [PMID: 23322957 DOI: 10.5455/aim.2012.20.72-84]

91 **Bashour H**, Muhjazi G. Hepatitis B and C in the Syrian Arab Republic: a review. *East Mediterr Health J* 2016; **22**: 267-273 [PMID: 27432409 DOI: 10.26719/2016.22.4.267]

Baishideng®

World Journal of

Gastroenterology

World Journal of Gastroenterology

Contents

Contents

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Nobuhiro Ohkohchi, MD, PhD, Professor, Department of Surgery, Division of Gastroenterology and Hepatobiliary Surgery and Organ Transplantation, University of Tsukuba, Tsukuba 305-8575, Japan

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 642 experts in gastroenterology and hepatology from 59 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

REVIEW

Non-pharmacological therapies for inflammatory bowel disease: Recommendations for self-care and physician guidance

Whitney Duff, Natasha Haskey, Gillian Potter, Jane Alcorn, Paulette Hunter, Sharyle Fowler

Whitney Duff, College of Kinesiology, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B2, Canada

Natasha Haskey, Irving K Barber School of Arts and Science, University of British Columbia-Okanagan, Kelowna, British Columbia V1V 1V7, Canada

Gillian Potter, Department of Psychology, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5A5, Canada

Jane Alcorn, College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5E5, Canada

Paulette Hunter, St Thomas More College, University of Saskatchewan, Saskatoon, SK S7N 0W6, Canada

Sharyle Fowler, Department of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W8, Canada

ORCID number: Whitney Duff (0000-0002-7236-9021); Natasha Haskey (0000-0001-7349-9445); Gillian Potter (0000-0003-2247-6360); Jane Alcorn (0000-0002-3473-2635); Paulette Hunter (0000-0003-1927-0433); Sharyle Fowler (0000-0002-2721-1591).

Author contributions: Duff W, Haskey N, and Potter G designed research, performed research, analysed data, and wrote the paper; Alcorn J, Hunter P, and Fowler S designed research and wrote the paper.

Correspondence to: Whitney Duff, MD, PhD, Doctor, College of Kinesiology, University of Saskatchewan, 87 Campus Drive, Saskatoon, Saskatchewan S7N 5B2, Canada. Whitney.duff@usask.ca
Telephone: +1-306-9661082
Fax: +1-306-9666464

Abstract

We performed a scoping review on sought-after complementary therapies for patients with inflammatory bowel disease (IBD), specifically diet, physical activity and exercise (PA/E), and psychotherapy. We aim to update patients with IBD on therapies for self-care and provide physicians with guidance on how to direct their patients for the management of IBD. A search of MEDLINE, EMBASE, and PUBMED was completed in Sept 2016. Studies on diet, PA/E, or psychotherapy in patients with IBD were included. Medical Subject Heading terms and Boolean operators were used. The search was limited to full-text English articles describing an adult population. This review included 67 studies: Diet ($n =$ 19); PA/E ($n =$ 19); and psychotherapy ($n =$ 29). We have made the following recommendations: (1) Diet: Consumption of diets rich in vegetables, fruit and soluble fiber may be beneficial in IBD. A trial of a low FODMAP diet can be considered in those patients with functional gastrointestinal symptoms. Restrictive diets are lacking in evidence and should be avoided; (2) PA/E: Regular low-moderate intensity activity, including cardiovascular and resistance exercise, has been shown to improve quality

of life (QOL) and may improve inflammation; and (3) psychotherapy: Therapies such as cognitive-behavioural interventions, mindfulness, hypnosis, and stress management have been shown to improve QOL, but evidence is limited on their impact on anxiety, depression, and disease activity. Overall, these complementary therapies are promising and should be used to treat patients with IBD from a more holistic perspective.

Key words: Scoping review; Inflammatory bowel disease; Diet; Exercise; Psychotherapy

Core tip: For diet, consumption of diets rich in vegetables, fruit and soluble fiber may be beneficial in inflammatory bowel disease. A low FODMAP diet can be considered in those patients with functional gastrointestinal symptoms. Restrictive diets are lacking in evidence and should be avoided. Regular low-moderate intensity activity has been shown to improve quality of life (QOL) and may improve inflammation. Therapies such as cognitive-behavioural interventions, mindfulness, hypnosis, and stress management have been shown to improve QOL, but limited evidence shown the impact on anxiety, depression, and disease activity.

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract and comprises two main forms: Crohn's disease (CD) and ulcerative colitis (UC). The symptoms of IBD are variable and may include diarrhea, vomiting, rectal bleeding, abdominal pain, weight loss, and malnutrition. Both CD and UC are characterized by periods of inflammation and remission, and the quality of life (QOL) of individuals with IBD may be severely impaired[1]. Medications treat flares in disease activity, and are usually required to help maintain remission. However, many IBD patients prefer a non-pharmacologic complementary or alternative approach to disease management[2].

Complementary alternative medicine (CAM) is identified as therapies that are either beyond Western allopathic medicine, not currently a standard therapy, or that are delivered by an alternative practitioner or by self-care[3]. Internationally, 30%-56% of IBD patients regularly use CAM with reasons for use including a yearning for a sense of control over the disease, ineffectiveness or adverse effects with conventional therapies,

perceived favorable safety profile, and desire for a more holistic approach[2,4-10]. Such therapies may improve subjective symptoms and QOL[11,12]. However, lack of communication between patient and doctor regarding CAM use can have serious consequences due to potential interactions and toxicities, unintentional non-adherence, or intentional dosage reduction of the pharmaceutical therapy by the patient[8-11].

Dietary manipulation, physical activity and exercise (PA/E), and psychological strategies are not mainstream therapies, however such therapies are clinically relevant because they combat high risk environmental factors (*i.e.*, poor nutrition, inactivity, and stress and anxiety) that negatively impact disease activity[2,13,14]. Unlike other lifestyle therapies such as smoking cessation, there is a lack of clear evidence-based literature that focuses on using diet manipulation, PA/E, and psychological interventions in IBD[2,7]. The concern is that self-sought CAM may lack an evidence-base or health professionals may fail to make recommendations about CAM leaving patients frustrated[2,15]. Clear evidence-based guidance to patients and health professionals on CAM would alleviate this problem. The primary purpose of this scoping review is to update patients with IBD on lifestyle therapies for self-care and provide physicians with guidance on how to direct their patients[15,16]. We include three lifestyle therapies: dietary manipulation, PA/E, and psychological interventions.

A scoping review of Ovid MEDLINE on the effects of diet, PA/E, and psychological interventions on IBD outcomes was performed. The database was searched using the Medical Subject Heading (MSH) terms, Boolean operators, and limitations as shown in Figure 1. Additional limitations applied to the diet section were enteral nutrition and parenteral nutrition, as the focus was on dietary patterns. Similar searches were further run *via* EMBASE: Excerpta Medica and EMBASE: Classic, PubMed, and PsychINFO.

Figure 2 shows the results of the Ovid MEDLINE search for diet, PA/E, and psychotherapy. After limitations were applied, a single expert reviewer for diet (NH), PA/E (WD), and psychotherapy (GP) screened results. Figure 3 shows the results of the EMBASE: Excerpta Medica and EMBASE: Classic search for additional articles. For diet, an additional 14 articles beyond those already included were identified *via* bibliographies or PubMed. For PA/E, an additional 5 articles beyond those already included were identified *via* bibliographies and no additional articles were identified *via* PubMed. For psychotherapy, an additional 5 articles beyond those already included were identified *via* bibliographies and no additional articles were identified *via* PubMed or PsychINFO.

DIET

Patients with IBD view diet modification as an important treatment modality to holistically manage their disease[17]. Several small scale diet studies demonstrated improved disease activity and prolonged time to re-
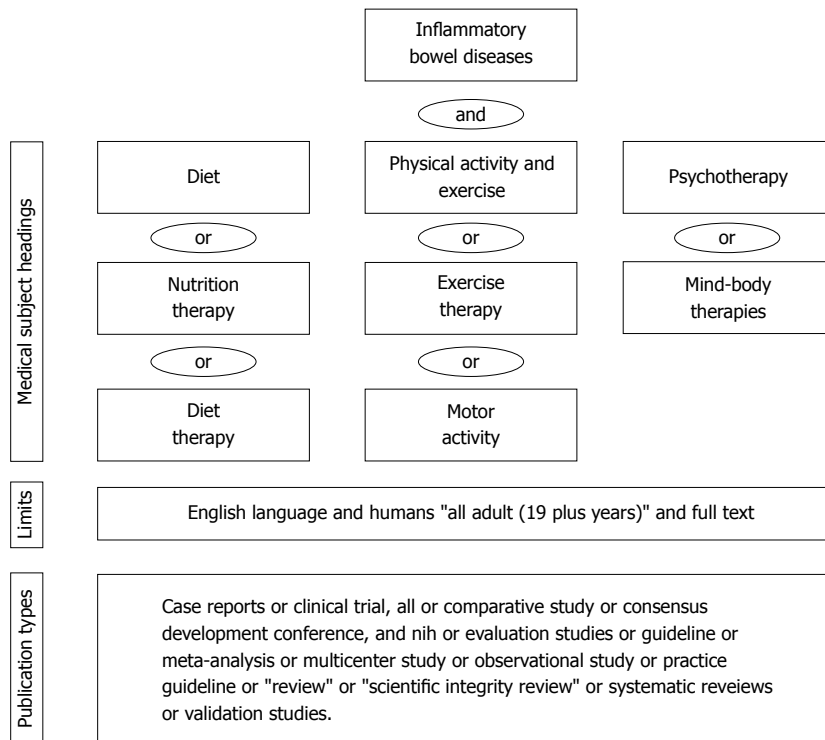
Figure 1 (flowchart)

Medical subject headings

Inflammatory bowel diseases

and

| Diet | Physical activity and exercise | Psychotherapy |

or ... *or* ... *or*

| Nutrition therapy | Exercise therapy | Mind-body therapies |

or ... *or*

| Diet therapy | Motor activity |

Limits: English language and humans "all adult (19 plus years)" and full text

Publication types: Case reports or clinical trial, all or comparative study or consensus development conference, and nih or evaluation studies or guideline or meta-analysis or multicenter study or observational study or practice guideline or "review" or "scientific integrity review" or systematic reveiews or validation studies.

Figure 1 Ovid MEDLINE literature search strategy. Similar searches were run within EMBASE: Excerpta Medica and EMBASE: Classic, PubMed, and PsychINFO databases.

Figure 2 (flowchart)

Initial search

| Diet (n = 236) | Physical activity and exercise (n = 83) | Psychotherapy (n = 439) |

Limited

| After limitations applied (n = 21) | After limitations applied (n = 14) | After limitations applied (n = 40) |

Screening

| After title and abstract review (n = 2) | After title and abstract review (n = 4) | After title and abstract review (n = 20) |

Figure 2 Ovid MEDLINE literature search results. After initial search and limitations, results were further screened by a single expert reviewer.

Figure 3 (flowchart)

Initial search

| Diet (n = 3330) | Physical activity and Exercise (n = 357) | Psychotherapy (n = 439) |

Limited

| After limitations applied (n = 183) | After limitations applied (n = 85) | After limitations applied (n = 58) |

Screening

| After title and abstract review (n = 3 additional articles) | After title and abstract review (n = 10 additional articles) | After title and abstract review (n = 4 additional articles) |

Figure 3 EMBASE: Excerpta Medica and EMBASE: Classic literature search results. After initial search and limitations, results above and beyond those already identified *via* Ovid MEDLINE search were further screened by a single expert reviewer.

lapse[18-25]. However, the efficacy of many of these interventions has not been examined rigorously. Regardless, reports indicate up to 68% of patients restrict their diet in an attempt to control symptoms and avoid exacerbation of the disease[17,26]. Additionally, th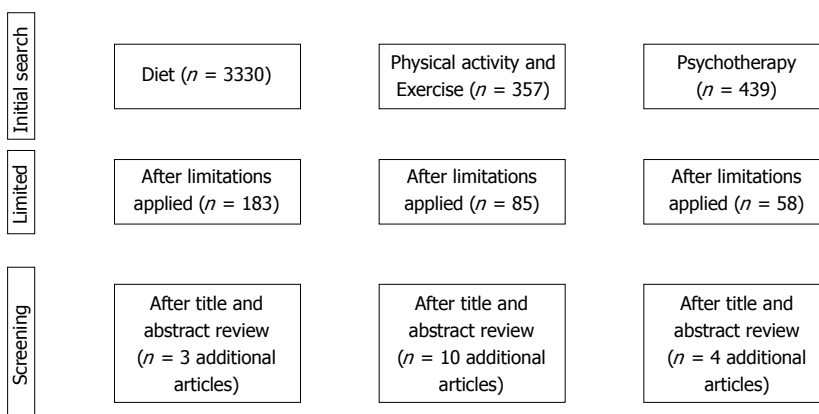e lack of clinical nutrition practice guidelines leaves health care practitioners with little direction on how to counsel their patients in this area. Patients then seek diet advice from the internet or patient support groups, rather than from their health care team[27]. The best available evidence for diet management of IBD is described below.

Fiber
Standard diet therapy as recommended by health professionals includes a low-residue diet (< 10-15 g/d of fiber), especially in patients with risk for gastrointestinal obstruction[28,29]. Despite this, evidence for the efficacy of low-residue diets in IBD is lacking. Seventy patients with CD were randomized to a low-residue diet (~3 g/d of fiber) or regular diet (13 g/d)[18]. There was no difference in outcome between the two groups, including symptoms, need for hospitalization, need for surgery, new complications, nutritional status, or postoperative disease recurrence. This study concluded that lifting dietary restrictions, which results in a more appetizing and nutritious diet, neither causes symptomatic deterioration nor precipitates intestinal obstruction in CD.

A randomized controlled trial (RCT) in CD examined the effects of fiber-related dietary instructions specifying daily consumption of whole wheat bran cereal and restriction of refined carbohydrates on QOL and gastrointestinal function (n = 7)[21]. Subjects in the intervention group were instructed to eat whole wheat bran cereal (½ cup) each day, to drink at least 48 ounces of unsweetened fluids each day, and reduce refined sugar. The study concluded that consuming a wheat bran inclusive diet was well-tolerated and there were no negative effects reported. The wheat bran group had increased scores on the Inflammatory Bowel Disease Questionnaires (IBDQ) over time (indicating greater improvement in QOL) than those in the control group (P = 0.028). The partial Harvey Bradshaw Index (HBI) scores decreased significantly over time in the active wheat bran intervention group, demonstrating improved GI function compared to participants in the control group (P = 0.008). Contrary to these results, a study in UC (n = 29) where subjects increased their fiber intake through consumption of whole wheat bread, vegetables, and a supplement of 25 g bran, found no evidence to support the concept that a high-fiber diet is of value in maintaining clinical remission in patients with UC[30].

A prospective longitudinal cohort study of patients with CD (n = 1130) and UC (n = 489) examined the association between fiber exposure and the risk of disease flare[31]. Patients with CD whose median intake of fiber was 23.7 g per day were ~40% less likely to experience a flare at six months as compared with those whose median consumption was 10.4 g of fiber per day. Fiber intake among patients with UC did not have an impact. Interestingly, patients with CD who reported that they did not avoid high-fiber foods had a 40% lower likelihood of flare than those who avoided high-fiber foods [adjusted OR: 0.59, 95%CI (0.43-0.81)].

A recent meta-analysis examining the role of fiber in the maintenance of remission in IBD, indicates weak evidence for the efficacy of fiber in improving disease outcomes in UC[32]. The effect of fiber in CD has not been clearly delineated, however studies do not show an increased risk of flaring or complications among patients with IBD consuming higher doses of fiber, suggesting that fiber is safe to consume in IBD[33].

Fat/animal protein
The search identified no randomized intervention trials that have specifically investigated overall dietary fat intake or animal protein intake in maintenance of remission in IBD. A prospective cohort study of 183 patients with UC found high intake of meat and meat products (processed meats) predicted an increased likelihood of relapse (OR: 5.19, 95%CI: 2.1-12.9)[34]. An intervention trial in Japan that targeted dietary fat intake by increasing the n-3 fatty acids while concurrently reducing the n-6 fatty acids found that among all IBD patients (CD and UC), participants with a greater n-3/n-6 fatty acid ratio reported higher rates of maintenance of remission (P < 0.001)[35]. In order to achieve this, patients were prohibited from consuming vegetable oil, margarine, salad dressings, mayonnaise and food cooked in vegetable oil, and intake of fish oils (EPA + DHA) was increased to 1700 mg. This study concluded that altering the fatty acid composition of the diet may be useful in maintaining remission in IBD.

Plant-based diets
Vegetarianism is a plant-based dietary pattern that includes different types of diets that vary depending on whether they include animal-derived foods such as milk and eggs[36]. Consumption of a vegetarian diet is associated with a number of health benefits such as a significantly lower risk for ischemic heart disease mortality (29%), overall cancer incidence (18%)[37], and lower risk of developing Type 2 Diabetes than non-vegetarians[38]. A semi-vegetarian diet (SVD) was evaluated prospectively for two years in patients with CD[19]. Remission rate was 94% (n = 15/16) in the subjects consuming a SVD *vs* 25% the omnivorous diet (P = 0.0003). The SVD was a lacto-ovo vegetarian diet that emphasized consumption of grains, daily intake of brown rice, vegetables, and fruits, while limiting intake of animal sources of protein. Fish was allowed once weekly, meat once every 2 wk and eggs/dairy products were allowed without limitation. Foods believed to be risk factors for IBD such as sweets, bread, cheese, margarine, fast foods, carbonated beverages, and juices were discouraged. The concentration of C-reactive protein (CRP) was normal at the final visit in more than half of the patients in remis-

sion on a SVD: The study authors predict that if a SVD is continued long-term and CRP levels are normalized patients are lower risk for relapse.

A Mediterranean diet pattern (MDP) is a widely accepted valid diet intervention able to produce clear benefits for the management of several pathologies and an overall reduction of mortality[39,40]. The MDP is rich in plant foods (cereals, fruits, vegetables, legumes, tree nuts, seeds and olives), with olive oil as the principal source of added fat, along with high to moderate intakes of fish and seafood, moderate consumption of eggs, poultry and dairy products (cheese and yogurt), low consumption of red meat, and a moderate intake of alcohol (mainly wine during meals)[41]. A study in CD demonstrated that subjects that followed a Mediterranean inspired diet for 6 wk showed a trend in reduction of inflammatory biomarkers (*e.g.*, CRP) and a trend towards normalization of the gut microbiota[20].

Comprehensive diet advice

A prospective RCT evaluated the impact of comprehensive dietary guidelines on the clinical course of disease and QOL in patients with UC (*n* = 122) over a 6-mo period[22]. Participants in the intervention group were provided dietary guidelines in the form of an educational booklet that recommended the following: eat little and often (four to six times a day), drink adequate fluids, decrease excess intake of saturated and trans fatty acids, decrease simple carbohydrates and avoid insoluble fiber, reduce red meat and processed meats and use probiotics. In addition, caffeine, alcohol and processed foods were to be avoided. A total of 69% of the participants in the group that received diet intervention found the advice to be helpful in managing their disease. There was a reduction in the Simple Clinical Colitis Activity Index (SCCAI) in the intervention group with an increase in the control group (*P* = 0.0108) suggesting symptomatic improvement. Although a trend towards clinical improvement was observed, this was not statistically significant.

Exclusion diets

A number of exclusion diets are gaining interest in IBD: the specific carbohydrate diet[23,42-44], IBD anti-inflammatory diet (IBD-AID)[24], gluten-free diet[45], and the low FODMAP diet[25,46,47]. Functional-like gastrointestinal symptoms (FGS) are common in IBD and are reported in 57% of patients with CD, and 33% of patients with UC despite no evidence of active inflammation[48]. A low FODMAP diet (LFD) has been proposed for the management of FGS in patients with well-controlled IBD (no active inflammation). This dietary intervention involves the restriction and systematic reintroduction of fermentable oligosaccharides (fructans, galacto-oligosaccharides), disaccharides (lactose), monosaccharides (fructose), and polyols (sorbitol, mannitol), collectively fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPs)[49]. A retrospective follow-up study based on patient-reported questionnaires evaluated the

LFD in patients with IBD (*n* = 49)[25]. Forty-two percent of subjects with IBD experienced full improvement with the greatest effect on bloating and abdominal pain. Twenty-four percent were completely asymptomatic on the diet, with 66% reporting normalization in stools. The effectiveness of the low FODMAP diet in clinical practice in patients with IBD and coexisting FGS found 78% of patients reported satisfactory relief of symptoms (*P* < 0.001) and significant decreases in severity of all symptoms (*e.g.*, abdominal pain, belching, bloating, flatulence, incomplete evacuation, nausea and heartburn)[46]. More patients reported normal-frequency stools while following the LFD. A pilot study found similar results, with the symptoms most likely to respond to a LFD being abdominal pain, diarrhea, bloating, and flatulence[47].

The literature search found no RCTs that have examined the effect of a gluten-free diet on maintenance of remission in IBD. A cross-sectional survey of patients with IBD (*n* = 314) found that 65.5% of patients that followed a gluten-free diet showed improvement in at least one clinical symptom and 38.3% indicated fewer or less severe flares[45].

The specific carbohydrate diet (SCD) is a novel diet approach that is gaining attention. Although the SCD is praised on multiple IBD patient web sites, it has been poorly studied in objective trials. It restricts complex carbohydrates and eliminates refined sugar from the diet, based on the rationale that the sugars and complex carbohydrates are malabsorbed and could cause alterations in microbiome composition, contributing to the intestinal inflammation of IBD[50]. In children, a retrospective study (*n* = 26) examined the SCD with concurrent medical therapy (*n* = 15) or diet alone (*n* = 11) for a duration of three to 48 mo (mean of 9.6 ± 0.1 mo)[43]. Ten additional subjects acted as controls. A comparative analysis of the patients on the SCD *vs* controls, revealed significant improvement in Pediatric CDAI, CRP, and calprotectin over time for both groups (*P* = 0.03, 0.03, and 0.03, respectively). Successful maintenance of remission with the SCD allowed some patients to discontinue medications and maintain disease control on the SCD alone. Although, the strongest efficacy for this diet comes from pediatric studies[43,44], one recent case series in adults has been completed[23]. Survey data was collected from 50 individuals that followed the SCD for a mean of 35.4 mo (range 1-216 mo). Thirty-three subjects (66%) noted complete symptom resolution at a mean of 9.9 mo (range 1 to 60 mo) after starting the SCD. Patients' self-report of the effectiveness of the SCD was rated as a mean of 91.3% effective in controlling acute flare symptoms (range = 30% to 100%) and a mean of 92.1% effective at maintaining remission (range = 53% to 100%). Subjects with colonic and ileocolonic CD reported the most benefit from this diet. An additional study conducted an online survey of 417 patients with IBD, and found that SCD had a positive effect, with symptoms progressively decreasing over six months. In addition, over one third of participants felt the SCD helped them achieve remission[42]. Despite an increasing

number of studies reporting potential benefits of the SCD in IBD, the studies are largely based on patient reported data, are conducted retrospectively, are unblinded, and lack objective markers of inflammation. The effect of the SCD on the clinical course of IBD remains to be validated in large, randomized, intervention trials before it can be recommended in clinical practice.

The IBD-AID is a diet designed to reduce inflammation and provide nutrients to promote a beneficial gut microbiota[24]. The IBD-AID involves modification of certain carbohydrates (including lactose, wheat, corn and refined sugar), emphasizes the ingestion of pre- and probiotics (*e.g.*, soluble fiber, leeks, onions, and fermented foods), distinguishes between saturated, trans, mono- and polyunsaturated fats, and encourages an overall healthy diet pattern. Textures of the foods (*e.g.*, blenderized, ground, or cooked) are modified as needed[24]. After following the IBD-AID, 89% of (n = 24/27) subjects had a reduction in symptoms. All subjects that followed the diet for > 4 wk (n = 11) were able to downscale their medication regimen and all (100%) of the patients had their IBD symptoms reduced, including a reduction in bowel frequency. Of the CD patients (n = 8), HBI improved from an average of 11 at baseline (range 1 to 20) to 1.5 (range 0 to 3) after dietary intervention. The UC patients (n = 3) had a mean baseline Modified Truelove and Witts Severity Index of 7 at baseline, which improved to 0 after dietary intervention. Although results of this trial appear promising, a large prospective, intervention trial that measures biomarkers of inflammation in blood and stool, along with clinical disease activity, is necessary before the IBD-AID can be recommended to the IBD patient population.

Dairy products, especially milk, are commonly reported to cause symptoms in IBD patients. Patients that declare they are "dairy sensitive" may be sensitive to lactose, not the milk protein. To date, no published intervention trials exist on the response of dairy products in patients with UC. One study reported an increased prevalence of lactose-malabsorption in CD patients with small bowel involvement[51]. Advice by health professionals varies widely, however IBD patients should be permitted to consume dairy products if they are able to tolerate them and strict lactose exclusion, even though widely practiced, is not usually necessary[51,52]. Since the lactose content of dairy products varies, a completely lactose-free diet may not be necessary and some patients may tolerate a reduction in lactose intake.

PHYSICAL ACTIVITY AND EXERCISE

Reduced cardiovascular capacity[53], impaired muscle function[54,55], and secondary osteoporosis[56,57] has been reported in patients with IBD. Limited physical activity, particularly with CD[58], due to fatigue may partially explain these negative outcomes[54,59-61]. Conversely, regular physical activity seems to improve disease activity and fatigue in patients with IBD[59,62]. For example, regimented exercise has been shown to improve disease activity and QOL[63-65], as well as cardiovascular[64] and bone health[65]. The best available evidence for PA/E for IBD is described below.

Physical activity
Patients with CD in remission (n = 41, 37.6 ± 10.4 years) were assessed with sex- and age-matched healthy controls (n = 25, 37.0 ± 13.0 years) for muscle strength and endurance, as well as other objective measures of physical activity[54]. Patients with CD had significant impairment in functional capacity (-25.1%, P < 0.001) and isometric performance of the lower limbs (-24.8%, P < 0.001), assessed by the 12-repetition sit-up test and maximal isometric strength and endurance of leg extensors on leg press. Subjective physical activity questionnaire revealed CD patients scored higher (P < 0.05) for work physical activity [2.94 (0.80)] and lower for sport physical activity [2.41 (1.10)] than healthy controls [2.51 (0.44) and 2.66 (0.74)]. However, objective accelerometer measures of overall physical activity were similar. This study is the first known to report independent muscle performance impairment and objective measures of physical activity in CD patients[54].

Patients with CD (n = 17) were surveyed regarding physical activity and stress related to disease activity[66,67]. Instruments utilized included the International Physical Activity Questionnaire, the Perceived Stress Scale, and the Inflammatory Bowel Disease Questionnaire (IBDQ). There was a direct relationship between physical activity and QOL (r = -0.551, P = 0.022), with most participants (52.9%) performing "high levels" of physical activity. This study is limited by self-report and exclusion of medication history, as well as inability to determine cause[67]. In a prospective study, patients with CD (n = 1308) and UC (n = 549) in remission were assessed for activity levels at baseline *via* Godin leisure time activity index (LTAI) and disease activity six months from baseline[62]. Higher activity levels were associated with decreased risk of active disease in both CD (P = 0.01) or UC (P = 0.04). While large sample size and inclusion of both CD and UC patients is a major strength, the use of Godin LTAI may be a limit because the score produced does not allow determination of intensity or type of physical activity the patients were performing[62]. Finally, Crohn's and Colitis UK members were surveyed (n = 918), with 66% reporting current participation in exercise[68]. Although 72% reported that exercise "made them feel better", noting improvement in general well-being and confidence, 23% reported that exercise "made them feel worse" (6% did not answer)[68]. Of the observational studies, only one surveyed regarding exercise, defined as planned, structured, repetitive and purposive physical activity[68]. Patients with IBD may prefer "unstructured" physical activity over exercise, although limitations still exist to both[58]. Limitations to PA/E specific to IBD include pain (joint and abdominal), muscle weakness, health concerns, embarrassment, and toilet access; however, fatigue is by far the most prevalent limitation reported[68,69].

Fatigue

Some studies have explored whether PA/E influences the experience of fatigue in IBD. The van Langenberg group first assessed fatigue, anxiety, depression, and sleep quality in patients with CD [n = 181, 41 (18-68) years] and UC [n = 113, 50 (18-72) years] compared to healthy controls [n = 85, 38 (17-63) years] in a cross-sectional, longitudinal study[59]. Patients with CD and UC had significantly worse scores (*P* < 0.05) on all variables compared to healthy controls; however, there was a statistically non-significant trend toward worse Fatigue Impact Scale scores for the CD group in comparison to the UC group. The commencement of a regular exercise program generally led to greater improvements in overall fatigue (0.05 < *P* < 0.10) and was independently associated with concurrently active disease (OR: 3.36, 95%CI: 1.27-8.88) in the CD group. Thus, regular exercise may improve disease activity and ultimately improve fatigue experienced by patients with CD[59].

Second, muscle fatigue was measured and compared with self-reported fatigue in patients with CD [n = 27, 43 (38, 48) years] and sex- and age-matched healthy controls [n = 22, 43 (36-49) years][70]. The FIS physical component was significantly higher (*P* < 0.001) in CD patients [12 (9, 16)] *vs* healthy controls [3 (1, 4)], as was muscular fatigue of the knee extensors (*P* = 0.047) determined *via* the decrement in force production from maximal voluntary contraction on an isokinetic dynamometer [-5.2 (-8.2, -2.2) *vs* -1.3 (-3.9, 1.4) Nm/min, respectively]. Further, objective muscular fatigue was negatively correlated (*P* < 0.05) with subjective physical fatigue for both CD (r = -0.52) and healthy controls (r = -0.41). Finally, a physiological basis to fatigue in CD is suggested by significantly (*P* = 0.009) lower levels of serum anabolic hormone insulin-like growth factor-1 (IGF-1), and independent associations of low levels (*P* < 0.05) of IGF-1, vitamin D$_3$, and magnesium with muscular fatigue. This cross-sectional, observational study is the first known to demonstrate that subjective fatigue correlates with objective fatigue. Despite the ability to suggest a physiological basis of fatigue, the study design could not establish if fatigue is "an end-product of muscle dysfunction or a root cause in CD"[70].

Third, sleep and physical activity was assessed in CD [n = 49, 44 (21, 65 years)] compared to healthy controls [n = 30, 46 (21, 63 years)] *via* accelerometer[60]. There was a significant (*P* < 0.01) impairment of all physical activity in CD patients *vs* controls, including more time spent doing sedentary, light, or "lifestyle" (98% *vs* 96%) rather than moderate-vigorous (2% *vs* 4%) activities and reduced overall activity (1.32 × 10^6 *vs* 1.95 × 10^6 as per accelerometer counts). Further, lower physical activity was associated (*P* < 0.05) with self-reported global (OR: 5.7, 95%CI: 1.1-29.1) and physical fatigue (OR: 3.9, 95%CI: 1.1-14.5) in a bivariate analysis, as was longer duration since diagnosis (OR: 1.2, 95%CI: 1.03-1.5), systemic inflammation *via* C-reactive protein (OR: 22.6, 95%CI: 1.1-479.3), and low vitamin D3 defined as < 50 nmol/L (OR: 13.1, 95%CI: 2.5-68.7)

in a multivariate analysis. In this study, participants that did not achieve any bouts of moderate-vigorous activity were described as having "lower physical activity". This approach to classification may account for discrepancy with a previous study that found no difference between CD patients and healthy controls[70].

Recently, men and women with IBD in remission were recruited in a matched cross-sectional study[61]. Fatigued IBD patients (n = 10, 36.4 ± 12.3 years), defined as a Checklist Individual Strength-fatigue (CIS-fatigue) score of ≥ 35, were matched to non-fatigued IBD patients (n = 10, 38.2 ± 11.0 years; CIS-fatigue score of < 35). The fatigued group had a lower intensity of daily physical activity (Cohen's d effect size = 1.02; *P* = 0.037), and reduced cardiovascular fitness demonstrated *via* 6-min walk test (Cohen's d effect size = 0.80; *P* = 0.030)[61].

Patients with IBD can overcome fatigue to participate in PA/E. While unstructured physical activity may be preferred, those who participate in structured exercise do so 1-2 d per week at a moderate intensity[58,68]. Structured exercise, such as cardiovascular *vs* resistance training, may provide additional benefits as described below.

Exercise

Cardiovascular training: In a controlled trial men and women (n = 12, 38.3 ± 7.5 years) with CD performed low-intensity walking for 30 min, 3 d per week for 12 wk[64]. Low-intensity walking was defined as 60% of heart rate maximum (HR$_{max}$) determined using the Karvonen formula[71]. Predicted maximal oxygen uptake improved (*P* = 0.001), implying improved cardiovascular fitness. Further, general well-being, QOL, and perceived stress significantly improved [IBDQ (*P* = 0.01)] and IBD (Stress index *P* < 0.001)[64].

In a follow-up study men and women (n = 32) with CD were randomized to low-intensity walking (60% HR$_{max}$) for 30 min, 3 d per week for 3 mo (n = 16, 40.6 ± 11.7 years) or to non-exercise control group (n = 16, 37.0 ± 12.7 years)[65]. Exercise improved QOL and perceived stress [IBDQ (*P* < 0.05)] and IBD Stress Index (*P* < 0.05). Importantly, exercise participants had improved disease activity based on HBI post-intervention compared to baseline (*P* < 0.01) while the non-exercise group worsened (*P* = 0.04).

Recently, men and women (n = 30) with IBD were randomized (stratified by disease) to supervised moderate intensity "running" 3 d per week for 10 wk (n= 15, 39.7 ± 14.7 years) or control group (n = 15, 42.5 ± 13.9 years)[63]. Moderate intensity "running" was defined as a pace great enough to work up a sweat while maintaining ability to talk. Exercise participants improved on the social well-being sub-scale of the IBDQ (*P* = 0.026) compared to controls. This is the only clinical trial to assess objective markers of inflammation (*i.e.*, leukocytes, C-reactive protein, and fecal calprotectin), however no statistically significant changes were seen in these parameters[63].

Resistance training: In a RCT men and women (n =

117) with CD were randomized to low-impact home-based exercise program performed 2 d per week for 1 year (n = 53, 40.1 ± 12.6 years) or control group (n = 54, 41.2 ± 14.1 years)[66]. The exercise program was low-impact and progressive and targeted sites susceptible to secondary osteoporosis (hip and lumbar spine) and major muscle groups (core and lower body). Participants who were ≥ 80% compliant to the exercise improved (P = 0.02) percent change of areal bone mineral density (aBMD) at the greater trochanter, derived *via* dual energy X-ray absorptiometry, compared to the control group (95%CI: 0.86-8.48). Pearson's correlation (r) showed total number of exercise sessions completed in the 12 mo was significantly associated with percent change of aBMD at the femoral neck (r = 0.28, P = 0.04). Disease activity assessed by Simple Disease Activity Score did not improve. This study has numerous strengths, including a RCT design, and is the first and only to determine effects of resistance training on IBD *via* RCT. However, this study was limited by poor compliance[66], with division of participants into sub-groups based on compliance (low, medium, and high exercise) for further analysis. An exploratory follow-up determined that Acceptance of Illness at baseline was significantly different between sub-groups (F = 3.57, df = 55, P < 0.04) and was the best predictor of exercise uptake (r = 0.376, R^2 = 0.141)[72].

Female IBD patients (n = 148) were tested for quadriceps strength *via* electromechanical chair dynamometer before assigning a sub-sample (n = 19) to progressive resistance training of the quadriceps twice per week for 8 wk[73]. Intensity was set at 50% of maximum load for the first 4 wk and progressed 10% per week until 80% maximum was achieved. Quadriceps strength improved significantly after the 8 wk of training (pre = 28.5 ± 5.6 kg, post = 39.0 ± 3.2 kg), as did QOL assessed by the IBDQ (P < 0.001). This study is limited by the specificity of the training program (*i.e.,* quadriceps only)[73].

Exercise tolerance: For the most part exercise was well tolerated in the clinical trials[64-66], although one participant reported mild abdominal symptoms in response to exercise[63]. Given the current evidence base, these conclusions apply best to those with mildly active disease or disease in remission, and to those with CD rather than UC. While exercise did not exacerbate disease, inflammation[63] and disease activity[64,66] was not improved in most clinical trials. Lack of improvements in disease activity may reflect length of the intervention[63,64] or low compliance to the training programs[66]. However, QOL was improved.

PSYCHOTHERAPY

IBD has been associated with negative psychosocial outcomes, such as a poor QOL[74], anxiety and depression[75], and maladaptive coping[76,77]. Such outcomes may be a product of the disease[78] or contributing factors[79]. Given that psychological functioning can impact one's sense of well-being[80] and physical health[81,82], stress management and mental health treatment may be important for maintaining a good QOL and health in IBD. The evidence for the use of psychotherapeutic approaches used exclusively with people who have IBD is reviewed below.

Cognitive behavioural therapy
Cognitive behavioural therapy (CBT) is a collaborative problem-solving approach between the client and therapist, which aims to collaboratively identify, challenge, and modify maladaptive thoughts and behaviors[83]. At present, most psychotherapists utilize a cognitive-behavioral approach[84]. There is some evidence that CBT improves aspects of psychological health in IBD.

Following a CBT group with IBD patients (n = 28), IBD-related concerns significantly decreased from pre- to post-treatment and these changes were maintained at 9-mo follow-up[85]. Using the Rating Form of IBD Patient Concerns (RFIPC), concerns that were reduced included: Impact of disease (pre = 39.4, Post = 28.3, P = 0.007), complications (pre = 37.1, post = 23.6, P = 0.018), and sexuality (pre = 33.3, post = 22.4, P = 0.039). No significant reduction was found for body stigma (pre = 24.6, post = 20.9). IBD-related quality of life (IBDQ) has also shown improvement following CBT interventions; however, these changes appear to be short-lived[86-88]. Following CBT, patients with IBD report significantly lower substance use for coping[88], and women report less depressive coping styles at 9-mo follow up[85]. Results from treatment studies of comorbid IBD and mood or anxiety disorders are mixed; both computerized and face-to-face forms of CBT have been ineffective at reducing anxiety and depression[88-90], while a face-to-face intervention did lead to less depression symptoms in women[85].

Some research has explored whether the effects of CBT for patients with IBD depend on the level of psychiatric comorbidity. For instance, in one study, CBT led to short-term improvements in health-related QOL and coping abilities, but only in IBD patients with high mental health needs[90]. In another study, using a sample with low levels of psychological distress (n = 28), a piloted CBT intervention improved IBD-related quality of life (IBDQ) and self-efficacy (IBD self-efficacy scale), and decreased stress [perceived stress questionnaire (PSQ)][91]. Within some other studies showing a positive effect following CBT[85,86], psychiatric illness has been an exclusion criterion.

Physical health outcomes have also been examined in CBT studies for IBD. Piloting a RCT with both CD and UC patients (n = 174), the effects of CBT (face-to-face and computerized modalities) were examined[90]. Neither face-to-face nor computerized CBT had significant effects on disease activity (CDAI and SCCAI) throughout testing. Following treatment, a nearly equal number of patients in the treatment and control groups remained in remission (CBT: 73.2%, controls: 71.7%, P = 0.868). An

analysis of a 2-year follow-up on this study showed no impact on disease activity measures[89]. In another RCT, a computerized form of CBT for IBD patients (*n* = 199) failed to produce any significant effects on clinical index measures (HBI and SCCAI)[88]. In an uncontrolled trial, a 12-wk CBT group for IBD patients had no significant impact on clinical indexes (CDAI and CAI) or medication use[85].

Mindfulness-based therapies

Mindfulness originates from Eastern philosophies and was introduced to Western medicine by Jon Kabat-Zinn[92], who, in 1979, created the Mindfulness-Based Stress Reduction (MBSR) program for patients with chronic illness[93,94]. Mindfulness can be conceptualized as a non-judgmental, accepting, and intentional quality of attention towards unfolding present-moment experiences[92,94,95]. Mindfulness has been integrated into a number of psychotherapies supported by the American Psychological Association, including Acceptance and Commitment Therapy and Dialectical Behavioral Therapy.

Mindfulness appears to improve mental health outcomes in IBD. Improved outcomes include health-related QOL (IBDQ)[96], maintained QOL during flare ups[97], reduced depression (BDI)[98], long-lasting reductions in anxiety (STAI)[98], and use of problem-directed coping strategies [ways of coping checklist (WCC)][96]. IBD patients with high levels of stress and more severe abdominal symptoms may benefit more from mindfulness interventions than other IBD patients[96,97]. In a double-blind RCT with UC patients (*n* = 55), MBSR completers with a high severity of gastrointestinal symptoms at baseline demonstrated a greater reduction in the hindrance of bowel symptoms (IBDQ-bowel subscale) compared to MBSR participants with less severe symptoms - an effect not seen in the control group ($P < 0.001$)[97]. Berrill and colleagues found similar results in an IBD sample with high levels of stress (PSQ) and/or functional abdominal symptoms (irritable bowel syndrome symptom severity scale)[96].

Currently, there is limited evidence on the effectiveness of mindfulness-based therapies (MBT) in reducing disease activity in IBD, as measured by clinical indexes and biomarkers of inflammation[96,98]. However, the aforementioned RCT with UC patients demonstrated that MBSR completers with high stress levels (PSQ) exhibited fewer flare ups post-treatment compared to those in the control group with high stress levels ($P < 0.001$)[97]. Moreover, meditative techniques, including breathing, body postures, movement, and elements of mindfulness[99], were associated with reducing an inflammatory biomarker (CRP), in a group of 29 IBD patients. It remains unclear which intervention component(s) were responsible for the effects observed in the latter study.

Psychodynamic psychotherapy

The effect of short-term psychodynamic therapy combined with autogenic training was examined in a pro-

spective, multicenter RCT[100-102]. Three separate analyses were conducted to examine the influence of the intervention on psychosocial status[102], disease course[101], and health care use[100]. The therapy targeted relaxation, health behaviors, and coping. The sample comprised 108 CD patients across four treatment centers and patients completed an average of 47 wk of therapy.

In terms of psychological outcomes, no significant differences were found between the treatment group and controls with depression (BDI), anxiety (STAI), or QOL (German quality of life scale)[102]. On measures of disease course (CDAI and physical assessments), the therapy failed to show any promising results[101]. However, in analyzing the effect on health care use, the treatment group displayed significantly less hospital days and sick leave days[100].

Hypnosis

Hypnosis is described as "an ability to sustain a state of attentive, receptive, intense focal concentration with diminished peripheral awareness in response to a signal"[103]. There is evidence of its lasting effects on health and well-being in a variety of populations, with proposed mechanisms including immune and cognitive changes[104,105]. As a psychological intervention for IBD, the clinician delivers hypnotic suggestions to improve psychological and gastrointestinal health[104]. There is a lack of evidence for hypnosis as an effective intervention for anxiety and depression in IBD; however, there is some evidence of its effect on other psychological outcomes[104,106].

In a RCT with UC patients (*n* = 37), a 7-session hypnosis intervention produced the following effect sizes on QOL and self-efficacy measures: IBDQ-bowel subscale (*d* = 0.50), IBDQ-systemic health subscale (*d* = 0.48), IBDQ-total scale (*d* = 0.41), and the IBD self-efficacy scale (*d* = 0.34)[104]. However, there were no significant effects on perceived health competence (perceived health competence scale), stress (PSQ), IBD-related concerns (RFIPC), or medication adherence (medication adherence scale).

There have been observations of long-term reductions in disease severity (clinical assessments) and medication use following hypnosis, although this was found in an uncontrolled study with a small sample size (*n* = 13)[106]. In the aforementioned RCT of seven hypnosis sessions, those in the treatment condition had a significantly higher number of days until relapse compared to controls, $F = 4.8 (1, 48)$, $P = 0.03$, and 68% of patients in the treatment condition maintained remission (subjective markers of flare) for one year, whereas this was the case with only 40% of the controls, $\chi^2 (1) = 3.9$, $P = 0.04$[104]. Indeed, in an RCT in which hypnosis was used to treat UC (*n* = 25), biomarkers of autonomic activity and inflammation were significantly reduced within 30 min following a single 50-min session of hypnosis[107]. Specifically, hypnosis had significant effects on heartrate, reduced serum interleukin-6 levels by 53% (a measure of systemic inflammation), and

reduced three of six measures of rectal inflammation.

Stress management

Studies were classified as stress management interventions if they either provided education on stress and/or coping, or utilized relaxation techniques. Such interventions appear to positively impact psychological functioning in IBD. In most studies that assessed disease-related stress, ratings on the IBD-related QOL (IBDQ) and IBD-related stress (IBD stress index) were significantly reduced post-treatment[108-110]. Anxiety [hospital anxiety and depression scale (HADS) and STAI] is also effectively targeted by these interventions[111,112]. However, there is limited support for the use of stress management for IBD patients with comorbid depression[111-113].

Studies that assessed disease activity following stress management interventions show mixed results. Following a stress management program for IBD patients, significant reductions in CDAI scores were observed[109]. However, two recent studies showed no effect on disease activity (HBI, CAI, and CDAI)[108,112]. Stress management interventions might improve self-reported pain and other symptoms. For example, in three RCTs using relaxation training for IBD patients, significant reductions were found on the visual analogue scale (VAS)[110], McGill pain questionnaire and pain and distress scale[114], and symptom-monitoring diaries[115]. In contrast, in a study of IBD patients with low levels of disease activity, researchers failed to find any significant reductions on the VAS[113].

Supportive-expressive group therapy

In a prospective, uncontrolled pilot study, supportive-expressive group therapy was examined with IBD patients (n = 30)[116]. This therapy aimed to encourage self-expression and reduce feelings of isolation. On psychological measures, there were no significant changes in IBD-related concerns (RFIPC), IBD-related quality of life (IBDQ), or anxiety or depression (HADS) post-treatment, however, there were changes in coping (ways of coping inventory). That is, patients reported less use of maladaptive coping styles, including denial, suppression, and self-blame. On measures of IBD symptoms, no significant changes occurred post-treatment, despite a reduction in symptoms[116].

Solution-focused therapy

Vogelaar and colleagues assessed the value of solution-focused therapy (SFT) in improving fatigue in IBD in two separate studies[117,118]. SFT is a brief form of psychotherapy that focuses on the individual's adequate coping abilities[118]. In the first study, SFT was compared against problem-solving therapy and a control condition in CD patients with high levels of fatigue (n = 40). No significant differences were found on any of the psychological outcomes, which included measures of QOL (IBDQ and EuroQol-5D), and anxiety and depression (HADS). In

the most recent study of highly fatigued patients (n = 98), the authors found short-term therapeutic effects on QOL (IBDQ) (P = 0.02) and depression (HADS) (P = 0.03) following SFT; however, no significant differences in anxiety were found between the treatment group and controls. When assessing fatigue, the initial study failed to find significant differences in fatigue scores on the checklist of individual strength (CIS) between treatment groups and controls, however, a greater number of patients in the SFT condition showed improvements. In the latter study, patients were assessed on measures of physical health outcomes from post-treatment to 9-mo follow-up. A significantly greater number of individuals in the treatment group exhibited low fatigue scores (CIS) compared to controls post-treatment (P = 0.03); however, this effect was only sustained until 6-mo follow-up (P = 0.19). In this study, SFT did not have any significant effects on clinical indexes, sleep quality, medication use, or blood parameters.

Multi-component behavioral treatment

Multi-component behavioral treatment (MCBT) is a psychological intervention that combines muscle relaxation techniques, thermal biofeedback, training in coping, and education in IBD[119]. In an RCT using MCBT with 21 IBD patients, researchers examined outcomes on the BDI, STAI, IBD stress index, hassles scale, and psychosomatic symptom checklist[119]. The IBD Stress Index was the only psychological measure to significantly decrease from pre- to post-treatment in the MCBT condition (P < 0.05). On a self-report of IBD symptoms, a significantly greater number of controls reported symptom reductions than the treatment group following the study (82% *vs* 65%, χ^2 = 14.58, P < 0.01).

CONCLUSION

The non-pharmacological therapies reviewed are intended to be used as an adjunct to conventional therapies. Evidence-based recommendations for diet, PA/E, and psychotherapy are provided. However, recommendations may be limited to a particular subset of IBD patients (*e.g.*, those in remission).

The existing body of science on the impact of diet on IBD has its limitations. High quality, well-designed RCTs are lacking, as the majority of intervention trials are of short duration and lack objective measurements of disease activity. Researchers rely on patient self-reporting, which introduces bias. At this time, no single diet should be recommended for all patients with IBD; however, a diet rich in vegetables, fruit and fiber appears to be of benefit (Table 1). Of note, this is for patients with IBD who are in remission. There is an even greater paucity of evidence based information for dietary recommendations for patients with active disease. Unique dietary recommendations should be developed for each patient, depending on the course of the disease. Patients should be counseled regarding the lack of objective evidence for

Table 1 Summary of best available evidence for diet management of inflammatory bowel disease (remission)

Diet	Recommendation	Ref.
Fiber	There is a lack of evidence that fiber intake should be restricted in patients with IBD. Soluble fiber sources are encouraged[32]. A high fiber diet is likely safe in patients with IBD and may impart a weak benefit[33]. Cruciferous vegetables, fruit peels, nuts, seeds should be avoided in patients with known fibrostenotic stricture with obstructive symptoms[29,33]	Hwang *et al*[29], 2014 Wedlake *et al*[32], 2014 Kaplan *et al*[33], 2016
Dairy products	Strict avoidance of dairy products is not justified unless it clearly worsens diarrhea[52]. For patients who are lactose intolerant, it may still be possible to consume small amounts of dairy products with lower amounts of lactose such as fermented dairy products (yogurt and kefir), cottage cheese, butter and aged cheeses[29]	Richman/Rhodes[52], 2013
Low-FODMAP	A low-FODMAP diet may be worth trying in patients with IBD who have FGS such as bloating, abdominal pain or watery diarrhea that have persisted despite appropriate treatments[25,52]	Maagaard *et al*[25], 2016
Plant-based	Plant-based diets such as a lacto-ovo vegetarian diet or Mediterranean diet pattern may reduce gut inflammation in IBD[19,20]	Chiba *et al*[19], 2010 Marlow *et al*[20], 2013
Fat/animal protein	Avoidance of trans fatty acids from processed foods, margarine and fast foods may be warranted[34,52]. A diet low in animal fat, particularly from processed meat and red meat (< 2/wk) is encouraged[34,122]	Jowett *et al*[34], 2004 Owczarek *et al*[122], 2016
Specific carbohydrate/ IBD-AID/ gluten-free	The effect of the SCD, IBD-AID and the gluten-free diet on clinical course in IBD remains to be elucidated in future trials	

IBD: Inflammatory bowel disease; FODMAP: Fermentable oligosaccharides, disaccharides, monosaccharides, and polyols; SCD: Specific carbohydrate diet; AID: Anti-inflammatory diet.

Table 2 Summary of best available evidence for physical activity and exercise in inflammatory bowel disease

Type	Recommendation	Ref.
Physical activity	Lower levels of physical activity are associated with fatigue[60,61], systemic inflammation[60], and reduced cardiovascular fitness[61], whereas higher levels of physical activity may improve quality of life[67] and decrease risk of active disease[62]. Unstructured "lifestyle" and work-related physical activity may be preferential over exercise[58,60,67], although both are encouraged	van Langenberg *et al*[60], 2015 Vogelaar *et al*[61], 2015 Jones et al[62], 2015 Crumbock *et al*[67], 2009 Mack *et al*[58], 2011
Exercise	Structured exercise may improve overall fatigue[70], general well-being[68], and disease activity[70]. Thus, exercise, including cardiovascular and resistance training, should supplement "lifestyle" physical activity	Van Langenberg *et al*[70], 2014 Chan *et al*[68], 2013
Cardiovascular training	Low-moderate intensity cardiovascular training may improve cardiovascular fitness[64], disease activity[65], perceived stress[64,65], and quality of life[64,65], including social and general well-being[63,64]. Cardiovascular training should be incorporated into exercise regimen a minimum of 30 mins, 3 d per week	Klare *et al*[63], 2015 Loudon *et al*[64], 1999 Ng *et al*[65], 2007
Resistance training	Low-moderate intensity progressive resistance training may improve bone health[66], strength[73], and quality of life[73]. More evidence is needed for a specific prescription	Robinson *et al*[66], 1998 de Souza *et al*[73], 2014

the use of restrictive diets, as well as the potential risks these diets can have in patients who are predisposed to nutritional deficiencies. Patients should consult with a registered dietitian for individualized diet counseling to assess current intake, evaluate nutritional status, and assist in manipulating diet to prevent nutritional deficiencies and improve QOL.

Current exercise guidelines suggest cardiovascular training 20-60 min at 60%-90% maximum heart rate 3-5 d per week and resistance training a minimum of 2 d per week are outdated and not based on actual evidence in an IBD population[120,121]. Further research is needed to improve our understanding and to create development of specific exercise guidelines for patients with IBD. Evidence thus far emphasizes that patients should perform regular low-moderate intensity physical activity, including cardiovascular and resistance exercise, as it may positively impact QOL and inflammation (Table 2). Exercise also has the potential to prevent impaired cardiovascular and muscular function and secondary osteoporosis associated with IBD and is generally well

tolerated[59,62,69]. Patients should, however, consult a physician prior to commencing a regular exercise regime[120-122], as exercise tolerance may be lessened with active disease. Physicians should also discuss with patients the potential for PA/E related fatigue.

With respect to psychotherapy interventions in IBD, there has been more examination of CBT, MBT, hypnosis, and stress management than other interventions (Table 3). There is evidence that CBT and MBT improve coping and QOL, yet evidence for improvement of comorbid anxiety and depression in this population is limited. Many of the latter interventions were tailored to the needs of individuals with IBD, however, they were not designed to exclusively treat anxiety and depression. Thus, along with IBD-related adaptations, more symptom-specific interventions may be necessary to effectively target anxiety and depression in this population. CBT and MBT are also seemingly limited in their impact on disease activity; longer follow up assessments may be necessary, as health benefits from stress reduction may take time. Hypnosis in IBD has demonstrated

Table 3 Summary of best available evidence for psychological interventions in inflammatory bowel disease

Intervention	Recommendation	Ref.
Cognitive behavioural therapy	May be useful for developing adaptive coping skills[85,88,90], reducing IBD-related stress[85], and improving quality of life[86-88,90] Therapeutic gains are observed in individuals with varying degrees of distress.[85,86,90,91] Outcomes on anxiety and depression are inconsistent[85,86,88-90]	Mussell *et al*[85], 2003 Díaz-Sibaja *et al*[86], 2009 Keefer *et al*[87], 2012a McCombie *et al*[88], 2016 Mikocka-Walus *et al*[89], 2016 Mikocka-Walus *et al*[90], 2015 Keefer *et al*[91], 2012
Mindfulness-based therapies	Could foster adaptive coping[96] and maintain quality of life during flare ups, particularly among individuals with moderate-severe distress or abdominal symptoms[96,97]. The evidence for managing anxiety and depression[98], as well as disease activity[97,99], is limited.	Berrill *et al*[96], 2014 Jedel *et al*[97], 2014 Schoultz *et al*[98], 2015 Gerbarg *et al*[99], 2015
Hypnosis	Demonstrates the most promise for managing disease activity[104,106,107]	Keefer *et al*[104], 2013 Miller/Whorwell[106], 2008 Mawdsley *et al*[107], 2008
Stress management	These interventions appear to target anxiety[110-112], reduce IBD-related stress[109,110], and improve quality of life[108,110]. There is some support for managing pain[110,114,115]	Boye *et al*[108], 2011 Milne *et al*[109], 1986 Mizrahi *et al*[110], 2012 Larsson *et al*[111], 2003 Smith *et al*[112], 2012 Shaw/Ehrlich[114], 1987 Garcia-Vega/Fernandez-Rodriguez[115], 2004
Psychodynamic psychotherapy, supportive expressive group therapy, solution-focused therapy, multi-component behavioural treatment	Insufficient evidence to make recommendations at this time	

IBD: Inflammatory bowel disease.

some evidence for reducing disease activity; however, there is no evidence of its ability to decrease anxiety and depression. As with the CBT and MBT studies, the hypnosis interventions were not specific to anxiety and depression, which could explain such findings. Stress management interventions have led to lower disease-related stress, anxiety, and reports of pain, yet their influence on disease-activity remains mixed. Future research could explore modifications of evidence-based psychotherapies that specifically target anxiety and depression, and examine the application of these in IBD. Research into interventions showing promise for physical health improvements in IBD, such as hypnosis, could also continue being explored.

The non-pharmacological therapies reviewed are promising, but should be viewed as adjunct therapies to treat IBD patients holistically. Longer-term, carefully designed, non-pharmacological intervention trials are urgently needed to determine the optimal treatment modality for patients. Such trials should assess both subjective measures such as QOL, as well as objective measures of inflammation. While the recommendations provided are based on existing evidence, additional research on non-pharmacological approaches for the management of IBD is needed.

REFERENCES

1   **Crohn's and Colitis Foundation of Canada**. The impact of inflammatory bowel disease in Canada: 2012 Final Report and Recommendations [Internet]. 2012. Available from: URL: http://www.isupportibd.ca/pdf/ccfc-ibd-impact-report-2012.pdf

2   **Hilsden RJ**, Verhoef MJ, Best A, Pocobelli G. Complementary and alternative medicine use by Canadian patients with inflammatory bowel disease: results from a national survey. *Am J Gastroenterol* 2003; **98**: 1563-1568 [PMID: 12873578 DOI: 10.1111/j.1572-0241.2003.07519.x]

3   **Wieland LS**, Manheimer E, Berman BM. Development and classification of an operational definition of complementary and alternative medicine for the Cochrane collaboration. *Altern Ther Health Med* 2011; **17**: 50-59 [PMID: 21717826]

4   **Burgmann T**, Rawsthorne P, Bernstein CN. Predictors of alternative and complementary medicine use in inflammatory bowel disease: do measures of conventional health care utilization relate to use? *Am J Gastroenterol* 2004; **99**: 889-893 [PMID: 15128356 DOI: 10.1111/j.1572-0241.2004.30033.x]

5   **Lakatos PL**, Czegledi Z, David G, Kispal Z, Kiss LS, Palatka K, Kristof T, Nagy F, Salamon A, Demeter P, Miheller P, Szamosi T, Banai J, Papp M, Bene L, Kovacs A, Racz I, Lakatos L. Association of adherence to therapy and complementary and alternative medicine use with demographic factors and disease phenotype in patients with inflammatory bowel disease. *J Crohns Colitis* 2010; **4**: 283-290 [PMID: 21122517 DOI: 10.1016/j.crohns.2009.11.011]

6   **Leong RW**, Lawrance IC, Ching JY, Cheung CM, Fung SS, Ho JN, Philpott J, Wallace AR, Sung JJ. Knowledge, quality of life, and use of complementary and alternative medicine and therapies in inflammatory bowel disease: a comparison of Chinese and Caucasian patients. *Dig Dis Sci* 2004; **49**: 1672-1676 [PMID: 15573925 DOI: 10.1023/B:DDAS.0000043384.26092.f4]

7   **Li FX**, Verhoef MJ, Best A, Otley A, Hilsden RJ. Why patients with inflammatory bowel disease use or do not use complementary and alternative medicine: a Canadian national survey. *Can J Gastroenterol* 2005; **19**: 567-573 [PMID: 16151549 DOI:

10.1155/2005/943547]

8   **Mountifield R**, Andrews JM, Mikocka-Walus A, Bampton P. Doctor communication quality and Friends' attitudes influence complementary medicine use in inflammatory bowel disease. *World J Gastroenterol* 2015; **21**: 3663-3670 [PMID: 25834335 DOI: 10.3748/wjg.v21.i12.3663]

9   **Nguyen GC**, Croitoru K, Silverberg MS, Steinhart AH, Weizman AV. Use of Complementary and Alternative Medicine for Inflammatory Bowel Disease Is Associated with Worse Adherence to Conventional Therapy: The COMPLIANT Study. *Inflamm Bowel Dis* 2016; **22**: 1412-1417 [PMID: 27120570 DOI: 10.1097/MIB.0000000000000773]

10   **Weizman AV**, Ahn E, Thanabalan R, Leung W, Croitoru K, Silverberg MS, Steinhart AH, Nguyen GC. Characterisation of complementary and alternative medicine use and its impact on medication adherence in inflammatory bowel disease. *Aliment Pharmacol Ther* 2012; **35**: 342-349 [PMID: 22176478 DOI: 10.1111/j.1365-2036.2011.04956.x]

11   **Abitbol V**, Lahmek P, Buisson A, Olympie A, Poupardin C, Chaussade S, Lesgourgues B, Nahon S. Impact of complementary and alternative medicine on the quality of life in inflammatory bowel disease: results from a French national survey. *Eur J Gastroenterol Hepatol* 2014; **26**: 288-294 [PMID: 24407360 DOI: 10.1097/MEG.0000000000000040]

12   **Elsenbruch S**, Langhorst J, Popkirowa K, Müller T, Luedtke R, Franken U, Paul A, Spahn G, Michalsen A, Janssen OE, Schedlowski M, Dobos GJ. Effects of mind-body therapy on quality of life and neuroendocrine and cellular immune functions in patients with ulcerative colitis. *Psychother Psychosom* 2005; **74**: 277-287 [PMID: 16088265 DOI: 10.1159/000086318]

13   **Dam AN**, Berg AM, Farraye FA. Environmental influences on the onset and clinical course of Crohn's disease-part 1: an overview of external risk factors. *Gastroenterol Hepatol* (NY) 2013; **9**: 711-717 [PMID: 24764788]

14   **Fiocchi C**. Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology* 1998; **115**: 182-205 [PMID: 9649475 DOI: 10.1016/S0016-5085(98)70381-6]

15   **Oviedo J**, Farraye FA. Self-care for the inflammatory bowel disease patient: what can the professional recommend? *Semin Gastrointest Dis* 2001; **12**: 223-236 [PMID: 11726076]

16   **Cosnes J**. Smoking, physical activity, nutrition and lifestyle: environmental factors and their impact on IBD. *Dig Dis* 2010; **28**: 411-417 [PMID: 20926865 DOI: 10.1159/000320395]

17   **Limdi JK**, Aggarwal D, McLaughlin JT. Dietary Practices and Beliefs in Patients with Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2016; **22**: 164-170 [PMID: 26383912 DOI: 10.1097/MIB.0000000000000585]

18   **Levenstein S**, Prantera C, Luzi C, D'Ubaldi A. Low residue or normal diet in Crohn's disease: a prospective controlled study in Italian patients. *Gut* 1985; **26**: 989-993 [PMID: 2996991 DOI: 10.1136/gut.26.10.989]

19   **Chiba M**, Abe T, Tsuda H, Sugawara T, Tsuda S, Tozawa H, Fujiwara K, Imai H. Lifestyle-related disease in Crohn's disease: relapse prevention by a semi-vegetarian diet. *World J Gastroenterol* 2010; **16**: 2484-2495 [PMID: 20503448 DOI: 10.3748/wjg.v16.i20.2484]

20   **Marlow G**, Ellett S, Ferguson IR, Zhu S, Karunasinghe N, Jesuthasan AC, Han DY, Fraser AG, Ferguson LR. Transcriptomics to study the effect of a Mediterranean-inspired diet on inflammation in Crohn's disease patients. *Hum Genomics* 2013; **7**: 24 [PMID: 24283712 DOI: 10.1186/1479-7364-7-24]

21   **Brotherton CS**, Taylor AG, Bourguignon C, Anderson JG. A high-fiber diet may improve bowel function and health-related quality of life in patients with Crohn disease. *Gastroenterol Nurs* 2014; **37**: 206-216 [PMID: 24871666 DOI: 10.1097/SGA.0000000000000047]

22   **Kyaw MH**, Moshkovska T, Mayberry J. A prospective, randomized, controlled, exploratory study of comprehensive dietary advice in ulcerative colitis: impact on disease activity and quality of life. *Eur J Gastroenterol Hepatol* 2014; **26**: 910-917 [PMID: 24942954 DOI: 10.1097/MEG.0000000000000127]

23   **Kakodkar S**, Farooqui AJ, Mikolaitis SL, Mutlu EA. The Specific Carbohydrate Diet for Inflammatory Bowel Disease: A Case Series. *J Acad Nutr Diet* 2015; **115**: 1226-1232 [PMID: 26210084 DOI: 10.1016/j.jand.2015.04.016]

24   **Olendzki BC**, Silverstein TD, Persuitte GM, Ma Y, Baldwin KR, Cave D. An anti-inflammatory diet as treatment for inflammatory bowel disease: a case series report. *Nutr J* 2014; **13**: 5 [PMID: 24428901 DOI: 10.1186/1475-2891-13-5]

25   **Maagaard L**, Ankersen DV, Végh Z, Burisch J, Jensen L, Pedersen N, Munkholm P. Follow-up of patients with functional bowel symptoms treated with a low FODMAP diet. *World J Gastroenterol* 2016; **22**: 4009-4019 [PMID: 27099444 DOI: 10.3748/wjg.v22.i15.4009]

26   **Cohen AB**, Lee D, Long MD, Kappelman MD, Martin CF, Sandler RS, Lewis JD. Dietary patterns and self-reported associations of diet with symptoms of inflammatory bowel disease. *Dig Dis Sci* 2013; **58**: 1322-1328 [PMID: 22923336 DOI: 10.1007/s10620-012-2373-3]

27   **Hou JK**, Lee D, Lewis J. Diet and inflammatory bowel disease: review of patient-targeted recommendations. *Clin Gastroenterol Hepatol* 2014; **12**: 1592-1600 [PMID: 24107394 DOI: 10.1016/j.cgh.2013.09.063]

28   **Brown AC**, Rampertab SD, Mullin GE. Existing dietary guidelines for Crohn's disease and ulcerative colitis. *Expert Rev Gastroenterol Hepatol* 2011; **5**: 411-425 [PMID: 21651358 DOI: 10.1586/egh.11.29]

29   **Hwang C**, Ross V, Mahadevan U. Popular exclusionary diets for inflammatory bowel disease: the search for a dietary culprit. *Inflamm Bowel Dis* 2014; **20**: 732-741 [PMID: 24562173 DOI: 10.1097/01.MIB.0000438427.48726.b0]

30   **Davies PS**, Rhodes J. Maintenance of remission in ulcerative colitis with sulphasalazine or a high-fibre diet: a clinical trial. *Br Med J* 1978; **1**: 1524-1525 [PMID: 26448 DOI: 10.1136/bmj.1.6126.1524]

31   **Brotherton CS**, Martin CA, Long MD, Kappelman MD, Sandler RS. Avoidance of Fiber Is Associated With Greater Risk of Crohn's Disease Flare in a 6-Month Period. *Clin Gastroenterol Hepatol* 2016; **14**: 1130-1136 [PMID: 26748217 DOI: 10.1016/j.cgh.2015.12.029]

32   **Wedlake L**, Slack N, Andreyev HJ, Whelan K. Fiber in the treatment and maintenance of inflammatory bowel disease: a systematic review of randomized controlled trials. *Inflamm Bowel Dis* 2014; **20**: 576-586 [PMID: 24445775 DOI: 10.1097/01.MIB.0000437984.92565.31]

33   **Kaplan GG**. Fiber and the Risk of Flaring in Patients With Inflammatory Bowel Diseases: Lessons From the Crohn's and Colitis Foundation of America Database. *Clin Gastroenterol Hepatol* 2016; **14**: 1137-1139 [PMID: 27155554 DOI: 10.1016/j.cgh.2016.04.034]

34   **Jowett SL**, Seal CJ, Pearce MS, Phillips E, Gregory W, Barton JR, Welfare MR. Influence of dietary factors on the clinical course of ulcerative colitis: a prospective cohort study. *Gut* 2004; **53**: 1479-1484 [PMID: 15361498 DOI: 10.1136/gut.2003.024828]

35   **Uchiyama K**, Nakamura M, Odahara S, Koido S, Katahira K, Shiraishi H, Ohkusa T, Fujise K, Tajiri H. N-3 polyunsaturated fatty acid diet therapy for patients with inflammatory bowel disease. *Inflamm Bowel Dis* 2010; **16**: 1696-1707 [PMID: 20222122 DOI: 10.1002/ibd.21251]

36   **do Rosario VA**, Fernandes R, Trindade EB. Vegetarian diets and gut microbiota: important shifts in markers of metabolism and cardiovascular disease. *Nutr Rev* 2016; **74**: 444-454 [PMID: 27261272 DOI: 10.1093/nutrit/nuw012]

37   **Huang T**, Yang B, Zheng J, Li G, Wahlqvist ML, Li D. Cardiovascular disease mortality and cancer incidence in vegetarians: a meta-analysis and systematic review. *Ann Nutr Metab* 2012; **60**: 233-240 [PMID: 22677895 DOI: 10.1159/000337301]

38   **Satija A**, Bhupathiraju SN, Rimm EB, Spiegelman D, Chiuve SE, Borgi L, Willett WC, Manson JE, Sun Q, Hu FB. Plant-Based Dietary Patterns and Incidence of Type 2 Diabetes in US Men and Women: Results from Three Prospective Cohort Studies. *PLoS Med* 2016; **13**: e1002039 [PMID: 27299701 DOI: 10.1371/journal.pmed.1002039]

39   **Schwingshackl L**, Hoffmann G. Adherence to Mediterranean diet and risk of cancer: a systematic review and meta-analysis of

observational studies. *Int J Cancer* 2014; **135**: 1884-1897 [PMID: 24599882 DOI: 10.1002/ijc.28824]

40   **Schwingshackl L**, Missbach B, König J, Hoffmann G. Adherence to a Mediterranean diet and risk of diabetes: a systematic review and meta-analysis. *Public Health Nutr* 2015; **18**: 1292-1299 [PMID: 25145972 DOI: 10.1017/S1368980014001542]

41   **Bach-Faig A**, Berry EM, Lairon D, Reguant J, Trichopoulou A, Dernini S, Medina FX, Battino M, Belahsen R, Miranda G, Serra-Majem L; Mediterranean Diet Foundation Expert Group. Mediterranean diet pyramid today. Science and cultural updates. *Public Health Nutr* 2011; **14**: 2274-2284 [PMID: 22166184 DOI: 10.1017/S1368980011002515]

42   **Suskind DL**, Wahbeh G, Cohen SA, Damman CJ, Klein J, Braly K, Shaffer M, Lee D. Patients Perceive Clinical Benefit with the Specific Carbohydrate Diet for Inflammatory Bowel Disease. *Dig Dis Sci* 2016; **61**: 3255-3260 [PMID: 27638834 DOI: 10.1007/s10620-016-4307-y]

43   **Obih C**, Wahbeh G, Lee D, Braly K, Giefer M, Shaffer ML, Nielson H, Suskind DL. Specific carbohydrate diet for pediatric inflammatory bowel disease in clinical practice within an academic IBD center. *Nutrition* 2016; **32**: 418-425 [PMID: 26655069 DOI: 10.1016/j.nut.2015.08.025]

44   **Cohen SA**, Gold BD, Oliva S, Lewis J, Stallworth A, Koch B, Eshee L, Mason D. Clinical and mucosal improvement with specific carbohydrate diet in pediatric Crohn disease. *J Pediatr Gastroenterol Nutr* 2014; **59**: 516-521 [PMID: 24897165 DOI: 10.1097/MPG.0000000000000449]

45   **Herfarth HH**, Martin CF, Sandler RS, Kappelman MD, Long MD. Prevalence of a gluten-free diet and improvement of clinical symptoms in patients with inflammatory bowel diseases. *Inflamm Bowel Dis* 2014; **20**: 1194-1197 [PMID: 24865778 DOI: 10.1097/MIB.0000000000000077]

46   **Prince AC**, Myers CE, Joyce T, Irving P, Lomer M, Whelan K. Fermentable Carbohydrate Restriction (Low FODMAP Diet) in Clinical Practice Improves Functional Gastrointestinal Symptoms in Patients with Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2016; **22**: 1129-1136 [PMID: 26914438 DOI: 10.1097/MIB.0000000000000708]

47   **Gearry RB**, Irving PM, Barrett JS, Nathan DM, Shepherd SJ, Gibson PR. Reduction of dietary poorly absorbed short-chain carbohydrates (FODMAPs) improves abdominal symptoms in patients with inflammatory bowel disease-a pilot study. *J Crohns Colitis* 2009; **3**: 8-14 [PMID: 21172242 DOI: 10.1016/j.crohns.2008.09.004]

48   **Simrén M**, Axelsson J, Gillberg R, Abrahamsson H, Svedlund J, Björnsson ES. Quality of life in inflammatory bowel disease in remission: the impact of IBS-like symptoms and associated psychological factors. *Am J Gastroenterol* 2002; **97**: 389-396 [PMID: 11866278 DOI: 10.1111/j.1572-0241.2002.05475.x]

49   **Gibson PR**, Shepherd SJ. Evidence-based dietary management of functional gastrointestinal symptoms: The FODMAP approach. *J Gastroenterol Hepatol* 2010; **25**: 252-258 [PMID: 20136989 DOI: 10.1111/j.1440-1746.2009.06149.x]

50   **Penagini F**, Dilillo D, Borsani B, Cococcioni L, Galli E, Bedogni G, Zuin G, Zuccotti GV. Nutrition in Pediatric Inflammatory Bowel Disease: From Etiology to Treatment. A Systematic Review. *Nutrients* 2016; **8** [PMID: 27258308 DOI: 10.3390/nu8060334]

51   **Mishkin B**, Yalovsky M, Mishkin S. Increased prevalence of lactose malabsorption in Crohn's disease patients at low risk for lactose malabsorption based on ethnic origin. *Am J Gastroenterol* 1997; **92**: 1148-1153 [PMID: 9219788]

52   **Richman E**, Rhodes JM. Review article: evidence-based dietary advice for patients with inflammatory bowel disease. *Aliment Pharmacol Ther* 2013; **38**: 1156-1171 [PMID: 24102340 DOI: 10.1111/apt.12500]

53   **Otto JM**, O'Doherty AF, Hennis PJ, Mitchell K, Pate JS, Cooper JA, Grocott MP, Montgomery HE. Preoperative exercise capacity in adult inflammatory bowel disease sufferers, determined by cardiopulmonary exercise testing. *Int J Colorectal Dis* 2012; **27**: 1485-1491 [PMID: 22842663 DOI: 10.1007/s00384-012-1533-4]

54   **Wiroth JB**, Filippi J, Schneider SM, Al-Jaouni R, Horvais N, Gavarry O, Bermon S, Hébuterne X. Muscle performance in patients with Crohn's disease in clinical remission. *Inflamm Bowel Dis* 2005; **11**: 296-303 [PMID: 15735436 DOI: 10.1097/01.MIB.0000160810.76729.9c]

55   **Zaltman C**, Braulio VB, Outeiral R, Nunes T, de Castro CL. Lower extremity mobility limitation and impaired muscle function in women with ulcerative colitis. *J Crohns Colitis* 2014; **8**: 529-535 [PMID: 24315794 DOI: 10.1016/j.crohns.2013.11.006]

56   **Ezzat Y**, Hamdy K. The frequency of low bone mineral density and its associated risk factors in patients with inflammatory bowel diseases. *Int J Rheum Dis* 2010; **13**: 259-265 [PMID: 20704624 DOI: 10.1111/j.1756-185X.2010.01542.x]

57   . American Gastroenterological Association medical position statement: Celiac Sprue. *Gastroenterology* 2001; **120**: 1522-1525 [PMID: 11313323 DOI: 10.1053/gast.2001.24055]

58   **Mack DE**, Wilson PM, Gilmore JC, Gunnell KE. Leisure-time physical activity in Canadians living with Crohn disease and ulcerative colitis: population-based estimates. *Gastroenterol Nurs* 2011; **34**: 288-294 [PMID: 21814062 DOI: 10.1097/SGA.0b013e3182248732]

59   **van Langenberg DR**, Gibson PR. Factors associated with physical and cognitive fatigue in patients with Crohn's disease: a cross-sectional and longitudinal study. *Inflamm Bowel Dis* 2014; **20**: 115-125 [PMID: 24297056 DOI: 10.1097/01.MIB.0000437614.91258.70]

60   **van Langenberg DR**, Papandony MC, Gibson PR. Sleep and physical activity measured by accelerometry in Crohn's disease. *Aliment Pharmacol Ther* 2015; **41**: 991-1004 [PMID: 25783784 DOI: 10.1111/apt.13160]

61   **Vogelaar L**, van den Berg-Emons R, Bussmann H, Rozenberg R, Timman R, van der Woude CJ. Physical fitness and physical activity in fatigued and non-fatigued inflammatory bowel disease patients. *Scand J Gastroenterol* 2015; **50**: 1357-1367 [PMID: 25966749 DOI: 10.3109/00365521.2015.1046135]

62   **Jones PD**, Kappelman MD, Martin CF, Chen W, Sandler RS, Long MD. Exercise decreases risk of future active disease in patients with inflammatory bowel disease in remission. *Inflamm Bowel Dis* 2015; **21**: 1063-1071 [PMID: 25723616 DOI: 10.1097/MIB.0000000000000333]

63   **Klare P**, Nigg J, Nold J, Haller B, Krug AB, Mair S, Thoeringer CK, Christle JW, Schmid RM, Halle M, Huber W. The impact of a ten-week physical exercise program on health-related quality of life in patients with inflammatory bowel disease: a prospective randomized controlled trial. *Digestion* 2015; **91**: 239-247 [PMID: 25823689 DOI: 10.1159/000371795]

64   **Loudon CP**, Corroll V, Butcher J, Rawsthorne P, Bernstein CN. The effects of physical exercise on patients with Crohn's disease. *Am J Gastroenterol* 1999; **94**: 697-703 [PMID: 10086654 DOI: 10.1111/j.1572-0241.1999.00939.x]

65   **Ng V**, Millard W, Lebrun C, Howard J. Low-intensity exercise improves quality of life in patients with Crohn's disease. *Clin J Sport Med* 2007; **17**: 384-388 [PMID: 17873551 DOI: 10.1097/JSM.0b013e31802b4fda]

66   **Robinson RJ**, Krzywicki T, Almond L, al-Azzawi F, Abrams K, Iqbal SJ, Mayberry JF. Effect of a low-impact exercise program on bone mineral density in Crohn's disease: a randomized controlled trial. *Gastroenterology* 1998; **115**: 36-41 [PMID: 9649456 DOI: 10.1016/S0016-5085(98)70362-2]

67   **Crumbock SC**, Loeb SJ, Fick DM. Physical activity, stress, disease activity, and quality of life in adults with Crohn disease. *Gastroenterol Nurs* 2009; **32**: 188-195 [PMID: 19506435 DOI: 10.1097/SGA.0b013e3181a85d4b]

68   **Chan D**, Robbins H, Rogers S, Clark S, Poullis A. Inflammatory bowel disease and exercise: results of a Crohn's and Colitis UK survey. *Frontline Gastroenterol* 2014; **5**: 44-48 [PMID: 28839750 DOI: 10.1136/flgastro-2013-100339]

69   **DeFilippis EM**, Tabani S, Warren RU, Christos PJ, Bosworth BP, Scherl EJ. Exercise and Self-Reported Limitations in Patients with Inflammatory Bowel Disease. *Dig Dis Sci* 2016; **61**: 215-220 [PMID: 26254773 DOI: 10.1007/s10620-015-3832-4]

70   **van Langenberg DR**, Della Gatta P, Warmington SA, Kidgell DJ, Gibson PR, Russell AP. Objectively measured muscle fatigue in Crohn's disease: correlation with self-reported fatigue and associated factors for clinical application. *J Crohns Colitis* 2014; **8**: 137-146 [PMID: 23938210 DOI: 10.1016/j.crohns.2013.07.006]

71   **KARVONEN MJ**, KENTALA E, MUSTALA O. The effects of training on heart rate; a longitudinal study. *Ann Med Exp Biol Fenn* 1957; **35**: 307-315 [PMID: 13470504]

72   **Watters C**, Wright S J, Robinson R J, Krzywicki T, Almond L, Shevlin M, et al. Positive and negative wellbeing as predictors of exercise uptake in Crohn's disease: An exploratory study. *Psychol Health Med* 2001; **6**: 293-9 [DOI: 10.1080/13548500123365]

73   **de Souza Tajiri GJ**, de Castro CL, Zaltman C. Progressive resistance training improves muscle strength in women with inflammatory bowel disease and quadriceps weakness. *J Crohns Colitis* 2014; **8**: 1749-1750 [PMID: 25239575 DOI: 10.1016/j.crohns.2014.09.001]

74   **Gibson PR**, Weston AR, Shann A, Florin TH, Lawrance IC, Macrae FA, Radford-Smith G. Relationship between disease severity, quality of life and health-care resource use in a cross-section of Australian patients with Crohn's disease. *J Gastroenterol Hepatol* 2007; **22**: 1306-1312 [PMID: 17559375 DOI: 10.1111/j.1440-1746.2007.04930.x]

75   **Goodhand JR**, Wahed M, Mawdsley JE, Farmer AD, Aziz Q, Rampton DS. Mood disorders in inflammatory bowel disease: relation to diagnosis, disease activity, perceived stress, and other factors. *Inflamm Bowel Dis* 2012; **18**: 2301-2309 [PMID: 22359369 DOI: 10.1002/ibd.22916]

76   **Jordan C**, Sin J, Fear NT, Chalder T. A systematic review of the psychological correlates of adjustment outcomes in adults with inflammatory bowel disease. *Clin Psychol Rev* 2016; **47**: 28-40 [PMID: 27318795 DOI: 10.1016/j.cpr.2016.06.001]

77   **Kemp K**, Griffiths J, Lovell K. Understanding the health and social care needs of people living with IBD: a meta-synthesis of the evidence. *World J Gastroenterol* 2012; **18**: 6240-6249 [PMID: 23180944 DOI: 10.3748/wjg.v18.i43.6240]

78   **Kurina LM**, Goldacre MJ, Yeates D, Gill LE. Depression and anxiety in people with inflammatory bowel disease. *J Epidemiol Community Health* 2001; **55**: 716-720 [PMID: 11553654 DOI: 10.1136/jech.55.10.716]

79   **Maunder RG**, Levenstein S. The role of stress in the development and clinical course of inflammatory bowel disease: epidemiological evidence. *Curr Mol Med* 2008; **8**: 247-252 [PMID: 18537632 DOI: 10.2174/156652408784533832]

80   **Stein MB**, Heimberg RG. Well-being and life satisfaction in generalized anxiety disorder: comparison to major depressive disorder in a community sample. *J Affect Disord* 2004; **79**: 161-166 [PMID: 15023490 DOI: 10.1016/S0165-0327(02)00457-3]

81   **Mroczek DK**, Stawski RS, Turiano NA, Chan W, Almeida DM, Neupert SD, Spiro A 3rd. Emotional Reactivity and Mortality: Longitudinal Findings From the VA Normative Aging Study. *J Gerontol B Psychol Sci Soc Sci* 2015; **70**: 398-406 [PMID: 24170714 DOI: 10.1093/geronb/gbt107]

82   **Foster JA**, McVey Neufeld KA. Gut-brain axis: how the microbiome influences anxiety and depression. *Trends Neurosci* 2013; **36**: 305-312 [PMID: 23384445 DOI: 10.1016/j.tins.2013.01.005]

83   **Hofmann SG**, Asnaani A, Vonk IJ, Sawyer AT, Fang A. The Efficacy of Cognitive Behavioral Therapy: A Review of Meta-analyses. *Cognit Ther Res* 2012; **36**: 427-440 [PMID: 23459093 DOI: 10.1007/s10608-012-9476-1]

84   **Gaudiano BA**. Cognitive-behavioural therapies: achievements and challenges. *Evid Based Ment Health* 2008; **11**: 5-7 [PMID: 18223042 DOI: 10.1136/ebmh.11.1.5]

85   **Mussell M**, Böcker U, Nagel N, Olbrich R, Singer MV. Reducing psychological distress in patients with inflammatory bowel disease by cognitive-behavioural treatment: exploratory study of effectiveness. *Scand J Gastroenterol* 2003; **38**: 755-762 [PMID: 12889563 DOI: 10.1080/00365520310003110]

86   **Díaz Sibaja MA**, Comeche Moreno MI, Mas Hesse B. [Protocolized cognitive-behavioural group therapy for inflammatory bowel

disease]. *Rev Esp Enferm Dig* 2007; **99**: 593-598 [PMID: 18052663 DOI: 10.4321/S1130-01082007001000006]

87   **Keefer L**, Doerfler B, Artz C. Optimizing management of Crohn's disease within a project management framework: results of a pilot study. *Inflamm Bowel Dis* 2012; **18**: 254-260 [PMID: 21351218 DOI: 10.1002/ibd.21679]

88   **McCombie A**, Gearry R, Andrews J, Mulder R, Mikocka-Walus A. Does Computerized Cognitive Behavioral Therapy Help People with Inflammatory Bowel Disease? A Randomized Controlled Trial. *Inflamm Bowel Dis* 2016; **22**: 171-181 [PMID: 26360545 DOI: 10.1097/MIB.0000000000000567]

89   **Mikocka-Walus A**, Bampton P, Hetzel D, Hughes P, Esterman A, Andrews JM. Cognitive-Behavioural Therapy for Inflammatory Bowel Disease: 24-Month Data from a Randomised Controlled Trial. *Int J Behav Med* 2017; **24**: 127-135 [PMID: 27432441 DOI: 10.1007/s12529-016-9580-9]

90   **Mikocka-Walus A**, Bampton P, Hetzel D, Hughes P, Esterman A, Andrews JM. Cognitive-behavioural therapy has no effect on disease activity but improves quality of life in subgroups of patients with inflammatory bowel disease: a pilot randomised controlled trial. *BMC Gastroenterol* 2015; **15**: 54 [PMID: 25934170 DOI: 10.1186/s12876-015-0278-2]

91   **Keefer L**, Kiebles JL, Kwiatek MA, Palsson O, Taft TH, Martinovich Z, Barrett TA. The potential role of a self-management intervention for ulcerative colitis: a brief report from the ulcerative colitis hypnotherapy trial. *Biol Res Nurs* 2012; **14**: 71-77 [PMID: 21362636 DOI: 10.1177/1099800410397629]

92   **Kabat-Zinn J**. Mindfulness-based interventions in context: Past, present, and future. C*lin Psychol Sci Pract* 2003; **10**: 144-56 [DOI: 10.1093/clipsy.bpg016]

93   **Vøllestad J**, Nielsen MB, Nielsen GH. Mindfulness- and acceptance-based interventions for anxiety disorders: a systematic review and meta-analysis. *Br J Clin Psychol* 2012; **51**: 239-260 [PMID: 22803933 DOI: 10.1111/j.2044-8260.2011.02024.x]

94   **Ockene JK**, Ockene IS, Kabat-Zinn J, Greene HL, Frid D. Teaching risk-factor counseling skills to medical students, house staff, and fellows. *Am J Prev Med* 1990; **6**: 35-42 [PMID: 2383411]

95   **Christopher MS**, Charoensuk S, Gilbert BD, Neary TJ, Pearce KL. Mindfulness in Thailand and the United States: a case of apples versus oranges? *J Clin Psychol* 2009; **65**: 590-612 [PMID: 19358288 DOI: 10.1002/jclp.20580]

96   **Berrill JW**, Sadlier M, Hood K, Green JT. Mindfulness-based therapy for inflammatory bowel disease patients with functional abdominal symptoms or high perceived stress levels. *J Crohns Colitis* 2014; **8**: 945-955 [PMID: 24529603 DOI: 10.1016/j.crohns.2014.01.018]

97   **Jedel S**, Hoffman A, Merriman P, Swanson B, Voigt R, Rajan KB, Shaikh M, Li H, Keshavarzian A. A randomized controlled trial of mindfulness-based stress reduction to prevent flare-up in patients with inactive ulcerative colitis. *Digestion* 2014; **89**: 142-155 [PMID: 24557009 DOI: 10.1159/000356316]

98   **Schoultz M**, Atherton I, Watson A. Mindfulness-based cognitive therapy for inflammatory bowel disease patients: findings from an exploratory pilot randomised controlled trial. *Trials* 2015; **16**: 379 [PMID: 26303912 DOI: 10.1186/s13063-015-0909-5]

99   **Gerbarg PL**, Jacob VE, Stevens L, Bosworth BP, Chabouni F, DeFilippis EM, Warren R, Trivellas M, Patel PV, Webb CD, Harbus MD, Christos PJ, Brown RP, Scherl EJ. The Effect of Breathing, Movement, and Meditation on Psychological and Physical Symptoms and Inflammatory Biomarkers in Inflammatory Bowel Disease: A Randomized Controlled Trial. *Inflamm Bowel Dis* 2015; **21**: 2886-2896 [PMID: 26426148 DOI: 10.1097/MIB.0000000000000568]

100 **Deter HC**, Keller W, von Wietersheim J, Jantschek G, Duchmann R, Zeitz M; German Study Group on Psychosocial Intervention in Crohn's Disease. Psychological treatment may reduce the need for healthcare in patients with Crohn's disease. *Inflamm Bowel Dis* 2007; **13**: 745-752 [PMID: 17230495 DOI: 10.1002/ibd.20068]

101 **Jantschek G**, Zeitz M, Pritsch M, Wirsching M, Klör HU, Studt HH, Rasenack J, Deter HC, Riecken EO, Feiereis H, Keller W. Effect of psychotherapy on the course of Crohn's disease. Results

of the German prospective multicenter psychotherapy treatment study on Crohn's disease. German Study Group on Psychosocial Intervention in Crohn's Disease. *Scand J Gastroenterol* 1998; **33**: 1289-1296 [PMID: 9930393 DOI: 10.1080/00365529850172386]

102 **Keller W**, Pritsch M, von Wietersheim J, Scheib P, Osborn W, Balck F. Effect of psychotherapy and relaxation on the psychosocial and somatic course of Crohn's disease. *J Psychosom Res* 2004; **56**: 687-696 [DOI: 10.1016/S0022-3999(03)00122-3]

103 **Spiegel H**, Spiegel D. Trance and treatment: Clinical uses of hypnosis. Arlington, VA: American Psychiatric Publishing; 2008

104 **Keefer L**, Taft TH, Kiebles JL, Martinovich Z, Barrett TA, Palsson OS. Gut-directed hypnotherapy significantly augments clinical remission in quiescent ulcerative colitis. *Aliment Pharmacol Ther* 2013; **38**: 761-771 [PMID: 23957526 DOI: 10.1111/apt.12449]

105 **Gruzelier JH**. A review of the impact of hypnosis, relaxation, guided imagery and individual differences on aspects of immunity and health. *Stress* 2002; **5**: 147-163 [PMID: 12186693 DOI: 10.1080/10253890290027877]

106 **Miller V**, Whorwell PJ. Treatment of inflammatory bowel disease: a role for hypnotherapy? *Int J Clin Exp Hypn* 2008; **56**: 306-317 [PMID: 18569141 DOI: 10.1080/00207140802041884]

107 **Mawdsley JE**, Jenkins DG, Macey MG, Langmead L, Rampton DS. The effect of hypnosis on systemic and rectal mucosal measures of inflammation in ulcerative colitis. *Am J Gastroenterol* 2008; **103**: 1460-1469 [PMID: 18510607 DOI: 10.1111/j.1572-0241.2008.01845.x]

108 **Boye B**, Lundin KE, Jantschek G, Leganger S, Mokleby K, Tangen T, Jantschek I, Pripp AH, Wojniusz S, Dahlstroem A, Rivenes AC, Benninghoven D, Hausken T, Roseth A, Kunzendorf S, Wilhelmsen I, Sharpe M, Blomhoff S, Malt UF, Jahnsen J. INSPIRE study: does stress management improve the course of inflammatory bowel disease and disease-specific quality of life in distressed patients with ulcerative colitis or Crohn's disease? A randomized controlled trial. *Inflamm Bowel Dis* 2011; **17**: 1863-1873 [PMID: 21287660 DOI: 10.1002/ibd.21575]

109 **Milne B**, Joachim G, Niedhardt J. A stress management programme for inflammatory bowel disease patients. *J Adv Nurs* 1986; **11**: 561-567 [PMID: 3534041 DOI: 10.1111/j.1365-2648.1986.tb01288.x]

110 **Mizrahi MC**, Reicher-Atir R, Levy S, Haramati S, Wengrower D, Israeli E, Goldin E. Effects of guided imagery with relaxation training on anxiety and quality of life among patients with inflammatory bowel disease. *Psychol Health* 2012; **27**: 1463-1479 [PMID: 22646975 DOI: 10.1080/08870446.2012.691169]

111 **Larsson K**, Sundberg Hjelm M, Karlbom U, Nordin K, Anderberg UM, Lööf L. A group-based patient education programme for high-

anxiety patients with Crohn disease or ulcerative colitis. *Scand J Gastroenterol* 2003; **38**: 763-769 [PMID: 12889564 DOI: 10.1080/00365520310003309]

112 **Smith GD**, Watson R, Roger D, McRorie E, Hurst N, Luman W, Palmer KR. Impact of a nurse-led counselling service on quality of life in patients with inflammatory bowel disease. *J Adv Nurs* 2002; **38**: 152-160 [PMID: 11940128 DOI: 10.1046/j.1365-2648.2002.02159.x]

113 **Oxelmark L**, Magnusson A, Löfberg R, Hillerås P. Group-based intervention program in inflammatory bowel disease patients: effects on quality of life. *Inflamm Bowel Dis* 2007; **13**: 182-190 [PMID: 17206698 DOI: 10.1002/ibd.20061]

114 **Shaw L**, Ehrlich A. Relaxation training as a treatment for chronic pain caused by ulcerative colitis. *Pain* 1987; **29**: 287-293 [PMID: 3302842 DOI: 10.1016/0304-3959(87)90043-1]

115 **García-Vega E**, Fernandez-Rodriguez C. A stress management programme for Crohn's disease. *Behav Res Ther* 2004; **42**: 367-383 [PMID: 14998732 DOI: 10.1016/S0005-7967(03)00146-3]

116 **Maunder RG**, Esplen MJ. Supportive-expressive group psychotherapy for persons with inflammatory bowel disease. *Can J Psychiatry* 2001; **46**: 622-626 [PMID: 11582823 DOI: 10.1177/070674370104600706]

117 **Vogelaar L**, Van't Spijker A, Vogelaar T, van Busschbach JJ, Visser MS, Kuipers EJ, van der Woude CJ. Solution focused therapy: a promising new tool in the management of fatigue in Crohn's disease patients psychological interventions for the management of fatigue in Crohn's disease. *J Crohns Colitis* 2011; **5**: 585-591 [PMID: 22115379 DOI: 10.1016/j.crohns.2011.06.001]

118 **Vogelaar L**, van't Spijker A, Timman R, van Tilburg AJ, Bac D, Vogelaar T, Kuipers EJ, van Busschbach JJ, van der Woude CJ. Fatigue management in patients with IBD: a randomised controlled trial. *Gut* 2014; **63**: 911-918 [PMID: 23884638 DOI: 10.1136/gutjnl-2013-305191]

119 **Schwarz SP**, Blanchard EB. Evaluation of a psychological treatment for inflammatory bowel disease. *Behav Res Ther* 1991; **29**: 167-177 [PMID: 2021379 DOI: 10.1016/0005-7967(91)90045-5]

120 **Ball E**. Exercise guidelines for patients with inflammatory bowel disease. Gastroenterol Nurs Off J Soc Gastroenterol Nurses Assoc 1998; **21**: 108-111 [DOI: 10.1097/00001610-199805000-00002]

121 **Pérez CA**. Prescription of physical exercise in Crohn's disease. *J Crohns Colitis* 2009; **3**: 225-231 [PMID: 21172280 DOI: 10.1016/j.crohns.2009.08.006]

122 **Owczarek D**, Rodacki T, Domagała-Rodacka R, Cibor D, Mach T. Diet and nutritional factors in inflammatory bowel diseases. *World J Gastroenterol* 2016; **22**: 895-905 [PMID: 26811635 DOI: 10.3748/wjg.v22.i3.895]

Helicobacter pylori in human health and disease: Mechanisms for local gastric and systemic effects

Denisse Bravo, Anilei Hoare, Cristopher Soto, Manuel A Valenzuela, Andrew FG Quest

Denisse Bravo, Anilei Hoare, Cristopher Soto, Oral Microbiology Laboratory, Pathology and Oral Medicine Department, Faculty of Dentistry, Universidad de Chile, Santiago 8380492, Chile

Manuel A Valenzuela, Advanced Center for Chronic Diseases, Institute for Health-Related Research and Innovation, Faculty of Health Sciences, Universidad Central de Chile, Santiago 8380447, Chile

Andrew FG Quest, Advanced Center for Chronic Diseases, Center for Studies on Exercise, Metabolism and Cancer, Biomedical Science Institute, Faculty of Medicine, Universidad de Chile, Santiago 8380447, Chile

ORCID number: Denisse Bravo (0000-0002-5749-4086); Anilei Hoare (0000-0002-1617-6401); Cristopher Soto (0000-0002-8286-5512); Manuel A Valenzuela (0000-0002-8833-7382); Andrew FG Quest (0000-0001-8755-1590).

Author contributions: Bravo D, Hoare A, Soto C, Valenzuela MA and Quest AF contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version; Bravo D, Hoare A and Soto C were involved in the design of Table 1 and Figure 1.

Supported by Comisión Nacional de Investigación Científica y Tecnológica-Fondos de Financiamiento de Centros de Investigación en Áreas Prioritarias, No. 15130011 (to Quest AF); Fondo Nacional de Desarrollo Científico y Tecnológico, No. 1170925 (to Quest AF) and No. 1171615 (to Valenzuela MA); Fondo para la Investigación en Odontología Universidad de Chile, No. 17/020 (to Bravo D).

Abstract

Helicobacter pylori (*H. pylori*) is present in roughly 50% of the human population worldwide and infection levels reach over 70% in developing countries. The infection has classically been associated with different gastro-intestinal diseases, but also with extra gastric diseases. Despite such associations, the bacterium frequently persists in the human host without inducing disease, and it has been suggested that *H. pylori* may also play a beneficial role in health. To understand how *H. pylori* can produce such diverse effects in the human host, several studies have focused on understanding the local and systemic effects triggered by this bacterium. One of the main mechanisms by which *H. pylori* is thought to damage the host is by inducing local and systemic inflammation. However, more recently, studies are beginning to focus on the effects of *H. pylori* and its metabolism on the gastric and intestinal microbiome. The objective of this review is to discuss how *H. pylori* has co-evolved with humans, how *H. pylori* presence is associated with positive and negative effects

in human health and how inflammation and/or changes in the microbiome are associated with the observed outcomes.

Core tip: This review focuses on discussing how *Helicobacter pylori* (*H. pylori*) has co-evolved with humans, potential mechanisms that may explain both positive and negative correlations in population-based studies between *H. pylori* infection and the development of several diseases, as well as how inflammation and/or changes in the microbiome might be linked to the respective outcomes. Our analysis of the literature reveals that human infection by *H. pylori* has a longstanding history, whereby the consequences therefore are extremely complex and not always detrimental to the human host. Thus, future research should focus on determining how potentially beneficial consequences of this interaction could be promoted all the while preventing the disease-causing effects in humans.

INTRODUCTION

Helicobacter pylori (*H. pylori*) infects approximately 50% of the human population worldwide and the infection could reach more than 70% in developing countries[1,2] The consequences of infection have been associated with the development of different gastro-intestinal diseases, such as gastric ulcers, gastric cancer, mucosa-associated lymphoid tissue (MALT) lymphoma and biliary tract cancer[3]. Moreover, *H. pylori* infection has also been associated with extra gastric diseases, such us ischemic heart diseases[4], type 2 diabetes mellitus[5], anemia[6], adverse metabolic traits in obese subjects[7] and insulin resistance[8], to mention but a few.

Despite the existence of such associations, these diseases occur only in a small percentage of infected people, suggesting that the bacteria frequently persists in the human host without inducing any obvious signs of disease, and it has been suggested that *H. pylori* may also play a beneficial role in human health[9-14]. Indeed, recent studies indicate that the decreasing incidence of *H. pylori* in the developing world is paralleled by an increase in the incidence of allergies and autoimmune diseases[15]. Furthermore, the absence of *H. pylori* has

been linked to elevated incidence of diseases, such us multiple sclerosis and celiac disease, among others[16-18].

Several studies have focused on understanding the local and systemic effects triggered by this bacterium in order to understand how *H. pylori* can produce such diverse effects in the human host. One of the best-characterized mechanisms involved in such effects is likely to be the damage to the host induced by local and systemic inflammation[19]. However, more recently, studies are beginning to focus on the effects of *H. pylori* and its metabolism on the gastric and intestinal microbiome[20-23]. This emerging field of interest could explain, at least in part, the wide variety of effects that are currently attributed to the presence of *H. pylori* in the human body.

In this review, we discuss such gastric and extra-gastric effects of *H. pylori* and the possible mechanisms involved.

H. PYLORI AND THE CO-EVOLUTION WITH HUMANS: NEGATIVE AND POSITIVE EFFECTS

H. pylori is a Gram-negative bacterium whose presence in the stomach of infected individuals is linked to the development of several gastric diseases, such as chronic gastritis. Although it is estimated that 50% of the world population is infected by *H. pylori*, only a small percentage of infected patients develop more severe pathologies, such as ulcers (10%-15%) and stomach adenocarcinomas (less than 1%)[1,2], the latter representing 15.4% of the cancers produced by infectious agents worldwide in 2012[24]. These values suggest that while relevant to the development of severe diseases, including gastric cancer, this pathogen could also play other roles in the human host.

It is now well established that *H. pylori* has been a highly prevalent pathogen in humans for over sixty thousand years and that infection occurs mainly in the intimate family environment or through vertical transmission[25]. These continuous infections and contact with other bacterial strains promoted the existence of a large number of mutations and genetic variability among bacteria due to horizontal transfer of information[26-28]. The emerging differences have been characterized particularly with respect to geographic distribution and such studies have revealed that the observed genomic alterations allow *H. pylori* to survive in different microenvironments[29,30]. Moreover, these genetic modifications are thought to have lead to the emergence of less virulent strains, which may explain the low percentage of patients affected with serious pathologies, such as adenocarcinomas[31].

The events during human evolution associated with initial acquisition *H. pylori* are thought to have been the development of agrarian practices, as evidenced by the presence of DNA remnants in the *H. pylori* genome, such

as the *vir* genes of *Agrobacterium tumifaciens*[32]. Also population migration is likely to have contributed to the acquisition of genes or genomic islands important for *H. pylori* virulence. One of them is the *cagPAI* genomic island, where *cagA* is one of the most important virulence genes associated with an increase in the activation of pro-inflammatory pathways and the production of pro-inflammatory cytokines in the stomach mucosa[33,34]. According to genomic analyses of *H. pylori*, European colonization trips to South America may have contributed to the acquisition of *cagA* by indigenous people living in the Andes, who possessed *H. pylori* without a functional *cagA* gene[30,35]. Moreover, other studies analyzing South American populations, such as the Colombians, have determined that the mountain people with greater similarity to the native people of that region have a higher incidence of gastric cancer compared with residents of coastal towns, who were more strongly influenced by the colonization of African migrant populations. Currently, this sector of the population has a low incidence of gastric cancer associated with *H. pylori* infection[36,37].

However, it has been observed that although in certain populations positive CagA strains are more numerous (as in parts of Eastern Asia), there are specific characteristics that make it unlikely for them to spread to the rest of the world, such as Western countries, with fewer positive CagA strains. These observations suggest that "fitness traits" exist, which aid in the survival of the bacteria in different hosts, in addition to other well-known factors, like difference in the lifestyles, socioeconomic levels and diet of the host population[38]. Japan, for example, has the highest rate of gastric cancer worldwide, associated with the highest presence of CagA (57%); however a lower seroprevalence is observed compared to other populations[39]. In contrast, despite the high prevalence of *H. pylori* in India, a low rate of gastric cancer is registered (known as the "Indian Enigma"). One of the main hypotheses seeking to explain this enigma is that the higher rate of enteric infections in more poorly developed countries could boost the immune system and limit the consequences of *H. pylori* infection. In addition, the high diet content of peppers, which represent an important ingredient in the Indian diet, may protect against *H. pylori* infection[40]. In conjunction, these examples support the hypothesis that a variety of factors contribute to the fitness of *H. pylori* in different human host populations.

Therefore, bacterial and host fitness are very relevant in *H. pylori* infection. In particular, host genetics likely affect the progression of pathologies associated with *H. pylori* infection. Indeed, specific polymorphisms in genes coding for cytokines, such as IL-1β, IL-8, IL-10 and TNF-α, are associated with an increase in the pro-inflammatory responses, greater colonization and infection, as well as an increased risk of gastric cancer[41-45]. Also, polymorphisms in innate immunity genes, such as the toll-like receptor 4 (TLR4), are relevant because TLR4 is implicated as a receptor responsible for *H. pylori* induced signaling in gastric epithelial cells. Moreover, epigenetic changes due to hypermethylation in the promoter regions of tumor suppressor genes, such as *LOX, HAND1* and *APC,* and the alteration as well as deregulation of microRNAs (miRs) are associated with a higher prevalence of gastric cancer following *H. pylori* infection[46,47].

Despite clearly representing a human pathogen, evidence is available suggesting that this bacterium could also be considered a commensal bacteria in the human host. This notion is supported by the simple observation that the bacteria is present in so many individuals, yet generates relatively few symptoms or pathologies. This raises the issue as to whether to refer to *H. pylori* as a commensal or pathogen, because pathologies are likely not only to be associated with specific traits of *H. pylori*, but also with a series of specific conditions in the human host.

H. pylori has been found as part of the normal oral microbiota and part of the microbiota of the stomach in the absence of inflammation[48,49]. In addition, it has been reported that *H. pylori* infection is not associated with the onset of the gastric cancer, but rather with its recurrence and chronicity[50]. Moreover, the presence of *H. pylori* in the stomach microbiota may result in changes in the normal microbiota[21,23].

In this context, it is worth mentioning that several studies have attributed positive effects to *H. pylori* infection. These include the suppression of bacteria that cause tuberculosis (*Mycobacterium tuberculosis*), protection against asthma, Crohn's disease, esophageal reflux, diarrheal diseases, as well as esophageal cancer[9-14]. This controversy has led to the discussion whether eradication of *H. pylori* is recommendable to help restore the host's health status, or if alternative strategies should be developed to control virulence of the bacteria, thereby avoiding the appearance of ulcers and adenocarcinomas without eliminating the positive effects that this bacterium may have [51]. With this in mind, it is not surprising that *H. pylori* is so widely studied and considered a relevant target in many therapies.

H. PYLORI COLONIZATION: PROTECTION AND PROMOTION OF INFLAMMATORY DISEASES

Protective effect of H. pylori colonization
H. pylori infection is inversely associated with the development of some diseases, suggesting that the presence of these bacteria may also be beneficial to the host, as is the case for reducing the risk of obesity, childhood asthma, inflammatory bowel disease and celiac disease among others. In some cases the data available strongly support the notion that *H. pylori* presence is beneficial, while for others convincing data still remains at large (Figure 1 and Table 1).

Asthma: Asthma is characterized by a chronic hyper-responsiveness to specific and non-specific stimuli that

Table 1 Association of different diseases with the presence or absence of *Helicobacter pylori*

Disease	Association	Reference	Type/model of study	Sample size	Statistical analysis	*H. pylori* detection	Diagnosis of the pathology
Asthma	NA	Holster et al[53], 2012	Cohort study	545 childrens	Chi-square and t-tests. Univariate and multiple logistic regression analyses[a]	Serum anti-*H. pylori* immunoglobulin G, and CagA by ELISA	Positive diagnosed asthma by a medical questionnaire
	Positive	Den Hollander[54], 2016	Cohort study	3797 childrens	Chi-square test. Multivariate logistic regression analysis. Odd ratios 95%CI[b]	immunoglobulin G levels in serum	Positive diagnosed asthma by a medical questionnaire
	Inverse	Chen and Blaser[10], 2007	Cohort study	7663 adults	Unconditional logistic regression models. Odd ratios 95%CI[a]	immunoglobulin G levels in serum	Positive diagnosed asthma by a medical questionnaire
		Chen and Blaser[55], 2008	Case-control study	7412 individuals	Chi-square and t-tests[b]	Wampole ELISA	Positive diagnosed asthma by a medical questionnaire
		Sommer et al[57], 1998	Ex vivo study. Isolated T cells from gastric biopsy	Biopsies from 30 patients	Not mentioned	Histological detection	Endoscopic examination
		Bamford et al[56], 1998	Ex vivo study. Isolated T cells from gastric biopsy	n patients = 5, n control = 3	t-test[a]	Rapid urease test (RUT) or histopathology	-
		Oertli et al[59], 2013	In vivo study. C57BL/6 mice	60 mice	Chi-square. Mann Whitney U-test and Kruskal-Wallis test[a]	CFU from homogenised tissues	-
		De la Pena-Ponce et al[60], 2017	In vitro study. Airway epithelial cells	Between n = 3 to n = 10	One way ANOVA[a]	CagA detection by western blot	-
Inflammatory Bowel Disease	Inverse	Higgins et al[61], 2011	Meta-analysis and In vivo study. C57BL/6 mice	Between n = 3 to n = 9	Odd ratios 95%CI. P-value < 0.1. One way ANOVA and t-test[a]	Not mentioned	-
		Lord et al[62], 2018	Cross-sectional study	704 individuals	Odd ratios 95%CI[b]	Not mentioned	Not mentioned
		Castano-Rodriguez et al[63], 2017	Meta analysis	6130 patients and 74659 controls	Chi-square, t-test, fixed effect model and odd ratios 95%CI[b]	Histology, culture, rapid urease test, serology and/or urea breath test (UBT)	Not mentioned but differentiated among Crohn's disease, ulcerative colitis, IBD, and unclassified
Celiac Disease	Inverse	Lebwohl et al[65], 2013	Cross-sectional study	136179 individuals	Odd ratios and 95%CI[b]	Polyclonal immunochemical stain	Duodenal and gastric biopsies
		Narang et al[18], 2017	Cross-sectional study	324 childrens	Chi-square test or Fisher exact test, and odd ratio and 95%CI[a]	Giemsa staining and rapid urease test (RUT)	Serum levels of immunoglobulin A-tissue transglutaminase antibodies (IgA-tTG > 18 U/mL = CD+). Further analysis by upper gastrointestinal endoscopy for biopsies to confirm
		Lucero et al[17], 2017	Case-control study	66 patients and 50 controls	Chi-square test or Fisher exact test, and odd ratio and 95%CI[b]	Rapid urease test (RUT), histological evaluation and PCR	Duodenal histopathology and immunoglobulin A-tissue transglutaminase (IgA-tTG) serology
Multiple Sclerosis	Inverse	Yao et al[67], 2016	Meta analysis	1553 patients and 1253 controls	Chi-square test, odd ratio and 95%CI[b]	ELISA, immunofluorescence and latex agglutination tests	Not mentioned
		Jaruvongvanich.[68], 2016	Meta analysis	1902 individuals	Chi-square test, odd ratios, multivariant models and random-effect models[a]	Urea breath test (UBT), rapid urease test, PCR and ELISA	Diagnosed by neurologist using the McDonald criteria (based on clinical presentations, finding on magnetic resonance imaging and cerebrospinal fluid profile)
	Positive	Efthymiou et al[16], 2016	Cohort study	129 patients, 49 controls	Two-tailed t-test[a]	Serum anti-*H. pylori*, anti-VacA, anti-CagA, anti-Hsp60 ELISA	Not mentioned, but relapsing remitting MS (RRMS) and secondary progressive MS (SPMS) are differentiated

Disease	Result	Ref	Study type	Sample size	Statistical analysis	H. pylori detection	Medical records
Ischemic Heart Diseases	Positive	Liu et al[4], 2015	Meta analysis	5829 patients and ~16000 controls	Fixed and random effect models. Odd ratios and 95%CI. P-value = 0.06	Not mentioned	Myocardial perfusion imaging in patients with angina symptoms, chest pain, suspected CAD, cardiac related symptoms or risk stratications in patients with known CAD
		Shmuely et al[3], 2014	Cohort study	173 patients and 127 controls	Multivariate analysis. Odd ratios 95%CI. t-test and ANOVA[a]	Serum anti-H. pylori immunoglobulin G, and CagA by ELISA	Isquemic stroke
		Huang et al[72], 2014	Retrospective cohort study	17332 patients and 69328 controls	Chi-square and t-tests[a]	Not mentioned	
Anemia	Positive	Xu et al[6], 2017	Retrospective study	17791 individuals 7804 Hp positive	Chi-square and t-tests. Odd ratios 95%CI[a]	Serum anti-H. pylori immunoglobulin G and immunoglobulin M ELISA	Using haemoglobin level
		Flores et al[76], 2015	In vitro study. AGS human gastric adenocarcinoma cell line	n = 7 experiments	One way ANOVA or non-parametric t-test[a]	CFU from homogenised cells	-
		Flores et al[75], 2017	In vitro study. AGS human gastric adenocarcinoma cell line	n = 3 experiments	One way ANOVA or non-parametric t-test[a]	CFU from homogenised cells	-
		Kato et al[77], 2017	In vitro study. Isolated H. pylori strains from patients and controls (whole genome sequencing)	4 patients and 4 controls	t-test[a]	Biopsy directly inoculated in growth medium	Measuring serum iron and ferritin
NAFLD	Positive	Chen et al[17], 2017	Cohort study	2263 individuals	Chi-square and t-tests[a]	13C-labeled urea breath test (UBT)	Using the NALFD criteria suggested by the Chinese Liver Disease Association and Clinical Diagnosis Standards
Insulin resistance	Positive	Huang et al[78], 2009	In vitro study. C57BL/6 mice	n = 20	Chi-square test[a]	Gram staining, PCR and urease/catalase reactions	Histopathology and immunochemical analysis
		Aydemir et al[82], 2005	Cross-sectional Study	63 patients	t-test[a]	Giemsa staining	HOMA-IR
		Gunji et al[83], 2009	Cross-sectional Study	1107 participants (1008 IR- y 99 IR+)	Chi-square and t-tests[a]	Serum anti-H. pylori immunoglobulin G ELISA	HOMA-IR
		Chen et al[84], 2015	Cohort study	811 individuals	Chi-square or Fisher exact test[a]	Serum anti-H. pylori immunoglobulin G ELISA	HOMA-IR
		Polyzos et al[86], 2011	Meta analysis	2120 participants	t-test[a]	Gastric mucosa histologic examination for H. pylori presence, gastric mucosa rapid urease test (CLO test), serum H. pylori-specific immunoglobulin G antibody concentration (ELISA), serum H. pylori-specific immunoglobulin G antibody concentration (chemiluminescence)	HOMA-IR
		Yildirim et al[89], 2016	Cohort study	41 patients and 27 controls	t-test[a]	13C-labeled urea breath test (UBT) and gastroscopy	HOMA-IR
		Upala et al[8], 2016	Meta analysis	27544 participants	Chi-square. Odd ratio 95%CI[a]	Urea breath test (UBT), rapid urease test (RUT), PCR and ELISA	HOMA-IR

Disease	Type of association	Ref	Type of study and model	Sample size	Statistical analysis	Method for H. pylori detection	Previously diagnosed patients
Type 2 Diabetes Mellitus	NA	Anastasios et al[93], 2002	Cross-sectional study	67 patients and 105 controls	Chi-square. P-value < 0.05	Giemsa staining	Not mentioned
	Positive	Li et al[5], 2017	Meta analysis	57397 participants	Fixed and random effect models. Odd ratios and 95%CI[a]	13C or 14C urea breath test, stool antigen test, anti-H. pylori antibody, rapid urease test, histology or biopsy, culture	Using venous blood glucose values or currently taking diabetic medication
		Bener et al[91], 2007	Case-control study	210 patients	t-test[a]	Serum anti-H. pylori immunoglobulin G and immunoglobulin A ELISA	Fasting blood sugar (FBS) level, random blood sugar (RBS) level and hemoglobin A1c
		Devrajani et al[92], 2010	Case-control study	74 patients and 74 controls	Chi-square[a]	Stool H. pylori-antigen detection by Enzyme immunoassay (EIA)	
		Aslan et al[95], 2006	Cross-sectional Study	103 patients	t-test[a]	Rapid urease test (RUT) and histopathologic examination	Serum glucose concentration and serum insulin levels
		Nasif et al[96], 2016	Cross-sectional Study	100 patients	t-test, Manne Whitney U-test[a]	Serum anti-H. pylori IgG ELISA	Postprandial glucose level, glycated hemoglobin (HbA1c) and body mass index (BMI). Serum 8-OHdG and Ox-LDL
Periodontitis	Positive	Sujatha et al[106], 2015	Cohort study	40 patients	Fisher exact test[a]	Rapid urease test, histopathological examination	Periodontal examination
		Pei et al[192], 2015	Cross-sectional study	70 patients ans 70 controls	The ratios were compared using × 2 test and × 2 statistics was adjusted. t-test[a]	PCR for urease C gene	Probing depth (PD), plaque index (PI) and bleeding index (BI)
		Hu et al[107], 2016	In vitro study. THP-1 cells	28 samples from 14 patients	One way ANOVA[a]	real-time PCR	Probing depth (PD), plaque index (PLI), bleeding index (BI), attachment loss (AL)

Different studies were analyzed considering the association of Helicobacter pylori (H. pylori) presence/absence with the corresponding disease (type of association), the type of study and model, the sample size, the type of statistical analysis employed, the method used for H. pylori detection and the method used for the disease diagnosis. Positive and inverse associations are indicated. [a]P < 0.05, [b]P < 0.01. NA: Indicates no association; IBD: Inflammatory bowel disease; IR: Insulin resistance.

favor obstruction of the airways, characterized by increased serum immunoglobulin E (IgE) levels combined with infiltration of the lungs by eosinophils, mast cells and activated CD4+ T-cells, a process orchestrated by effector T-helper 2 cells, implying the participation of the cytokines IL-4, IL-13, IL-5 and IL-9 in these events[52]. Several studies have proposed an inverse association between the presence of H. pylori infection and asthma, although this association is still controversial. While Holster et al[53] showed in a cohort of 545 children that there are no significant differences in H. pylori prevalence between children with asthma (7.1% vs 9.4%), others have shown either positive or negative effects. Significantly higher prevalence of asthma was reported in H. pylori positive compared to H. pylori negative children, based on a cohort study of 3759 children[54]. In contrast, studies involving more than 7000 adults[10,55] showed that H. pylori presence and also the CagA protein were inversely correlated with the development of asthma. More recently, Miftahussurur et al[15] reviewed several studies, surveys, cohort studies and meta-analyses in different European counties and in the United States of America (USA), involving a large number of persons. They concluded that there is a significant but weak inverse correlation between H. pylori infection, allergies and asthma, suggesting that H. pylori infection may have a beneficial protective role against development of these diseases[15].

The proposed mechanism involves the bacterial induction of naïve T cells, mainly in T helper 1 (Th1) rather than helper 2 (Th2) subsets[56,57]. On the other hand, it has also been observed that T-regulatory (Treg) cells are increased in the gastric mucosa of H. pylori-infected humans[58]. Moreover, the H. pylori virulence factors γ-glutamyl transpeptidase and VacA, induced Treg cells in the mouse gastric mucosa, resulting in the development of tolerance and a reduction in allergic responses[59]. Also, in a recent study using infant and adult airway epithelial cells infected with H. pylori, IL-8 synthesis increased 4-fold in infant versus adult cultures, suggesting that the infant epithelium elicits a higher immune response than the adult tissue. This mechanism is mediated by the H. pylori type IV secretion system and stimulation of the p38 MAP kinase pathway[60], and VacA was found to potentially also contribute to this mechanism.
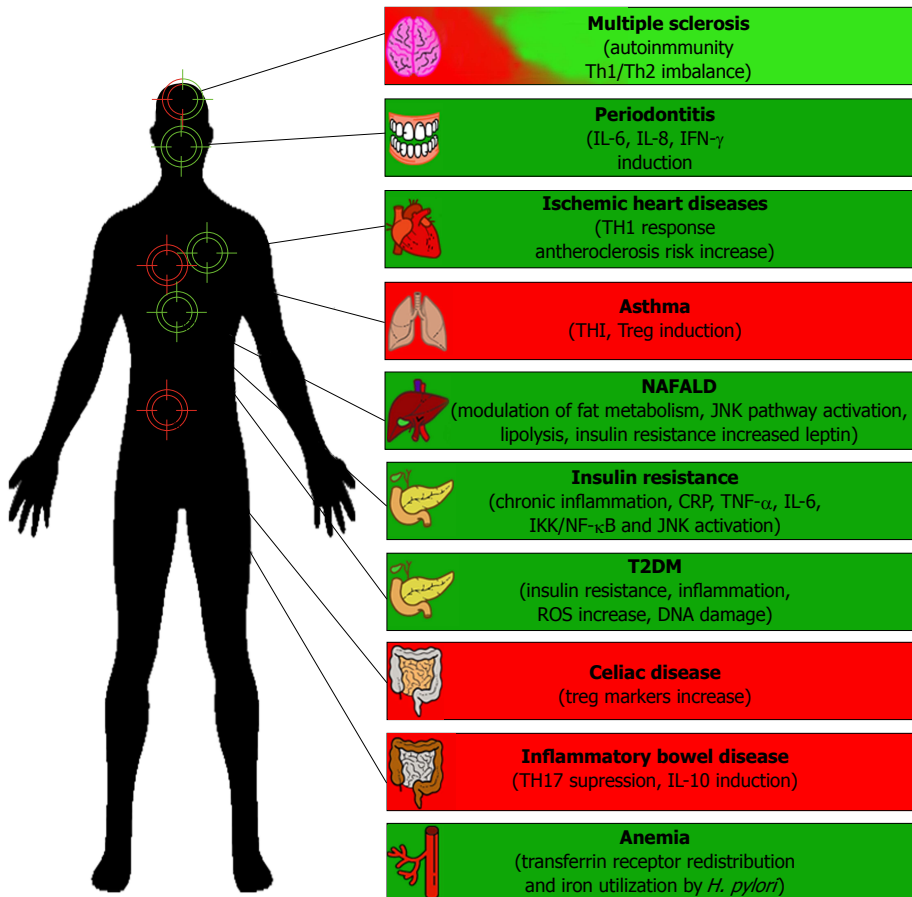
Figure 1 *Helicobacter pylori* **and extra-gastric disease association.** Green squares represent positive correlations between *Helicobacter pylori* (*H. pylori*) and the disease, while red squares represent inverse correlations between *H. pylori* and the disease. Multiple sclerosis is shown in red and green because there is information suggesting both positive and inverse correlations. NAFALD: Non-alcoholic fatty acid liver disease; T2DM: Type 2 diabetes mellitus.

Inflammatory bowel disease: Inflammatory bowel disease (IBD) is a chronic inflammatory intestinal disease that develops as the consequence of a deregulated immune response. Interestingly, several studies have sought to establish a relationship between *H. pylori* infection and IBD. Higgins *et al*[61] demonstrated the effect of gastric *H. pylori* colonization on a distant bacterial-host immune system interaction in an experimental model of colitis. Also, *H. pylori* was shown to suppress the Th17 response to *S.* Typhimurium infection, but did not alter the Th2 or Treg response. Moreover, the authors showed that the co-infection by *H. pylori*/S. Typhimurium decreases inflammation in both the cecum and the stomach and that *H. pylori* infection induces IL-10 in the mesenteric lymph nodes, suggesting an extra-gastric mechanism for immunomodulation. Also, IBD protection is suggested to be linked to the *cag*A-positive status of the strain[62]. More recently, a meta-analysis performed by Castaño-Rodríguez *et al*[63] also revealed that *H. pylori* may exert an immunomodulatory effect and thereby favor the development of IBD.

Celiac disease: Celiac disease (CD) is an autoimmune disease whose prevalence in the USA has increased up to 4-fold in the past 50 years[64]. A cross-sectional study of patients who underwent esophago-gastroduodendoscopy with analysis of gastric and duodenal biopsies during a 4.5-year period showed that *H. pylori* prevalence was lower in patients with CD (4.4%) than in those without CD[65], indicating an inverse association between CD and *H. pylori* infection.

In the same context, a recent study including 324 children with confirmed CD, the *H. pylori* prevalence was compared with a reference group of non-celiac children referred for endoscopy. The results showed that the prevalence of *H. pylori* in patients without CD was significantly higher[18], indicating that CD and gastric *H. pylori* infection are inversely correlated.

The mechanistic link between *H. pylori* infection and CD remains to be elucidated. However, recently, Lucero *et al*[17] demonstrated that infection by CagA positive *H. pylori* induced Treg markers and that this may be protective against CD progression.

Multiple sclerosis: Emerging evidence suggests that *H. pylori* may also be inversely associated with neuro-degenerative diseases. In this context, several studies have sought to establish an association between *H. pylori* infection and multiple sclerosis (MS), a chronic auto-immune, inflammatory and neurodegenerative disorder

of the central nervous system[66].

In a meta-analysis of nine studies involving 2806 cases (1553 patients with MS and 1253 controls), Yao *et al*[67] found that the prevalence of *H. pylori* infection in MS patients was lower than that in control groups. Another meta-analysis of six observational studies involving 1902 participants showed also a statistically significant lower prevalence of *H. pylori* infection in patients with MS[68].

In spite of these studies, Efthymiou *et al*[16] in a cohort study of 129 patients and 49 controls, showed that anti-*H. pylori* antibody titers were higher in 129 MS patients than in 48 healthy controls. Additionally, anti-*H. pylori* hsp 60 seropositivity correlated with age at disease onset, suggesting a possible role of this factor in the pathogenesis of MS[16].

In this context, it has been proposed that the inflammatory mediators induced by *H. pylori* infection might impact on the nervous system and induce damage[69]. Additionally, circulating pro-inflammatory cytokines, such as IL-17, and reactive oxygen species (ROS) can reach the CNS and induce damage[70]. Moreover, recent reports implicate the Galectin-3 receptor, a leptin receptor that is stimulated by *H. pylori*, in inducing a pro-inflammatory response *via* TLRs. Activation of these receptors in the CNS, triggers an inflammatory response mediated by interferon (IFN)-γ and TNF-α that is associated with neuro-pathophysiological changes [70].

Negative effect of H. pylori colonization

Despite these observations suggesting a protective role for *H. pylori* against several diseases, there is a large body of literature associating *H. pylori* infection with the development of gastric diseases, such as peptic ulcer diseases, gastric adenocarcinoma, MALT lymphoma and biliary tract[71]. Moreover, the positive correlations between *H. pylori* and disease conditions have also been noted for extra-intestinal diseases, such as dermatological diseases, heart diseases, obesity, anemia, insulin resistance and non-alcoholic fatty liver disease, among others (Figure 1 and Table 1). In most of these cases, disease development is associated with the chronic inflammatory response that the infection triggers in the host.

Ischemic heart diseases: *H. pylori* has been suggested to contribute to the development of coronary heart diseases (CAD). In a meta-analysis of 26 studies, including more than 20000 patients, Liu *et al*[4] observed a significant association between *H. pylori* infection and the risk of myocardial infarction. In the same context, Shmuely *et al*[3] in a cohort study of 173 patients and 127 controls, observed that *H. pylori* infection was significantly higher in CAD-positive patients than in CAD-negative subjects, suggesting a positive correlation between *H. pylori* seropositivity and CAD. In addition, in a retrospective cohort study by Huang *et al*[72], involving 17332 patients with *H. pylori* infection and 69328 randomly selected age- and gender-matched controls, a more specific association between chronic *H. pylori* infection and ischemic stroke was observed since patients diagnosed with *H. pylori* infection exhibited a higher incidence rate of ischemic stroke. Despite such observations suggesting that the presence of *H. pylori* favors the development of heart disease, the mechanisms involved remain to be determined. However, because chronic inflammation is believed to be associated with an increased risk of atherosclerosis[73], this may in an indirect manner explain the augmented risk of heart disease associated with *H. pylori* infection.

Anemia: An association between *H. pylori* infection and iron deficiency was proposed based on studies showing that for individuals with idiopathic iron deficiency anemia of unknown origin and no evidence of bleeding due to lesions, iron deficiency anemia was no longer observed in any of the follow-up examinations following eradication of *H. pylori*[74]. In a recent retrospective cohort study, Xu *et al*[6] evaluated the relationship between anemia and *H. pylori* infection in 17791 subjects. They observed a higher probability for anemia in *H. pylori* positive populations coincident with lower hemoglobin levels.

Recent studies suggest that the mechanism involves changes in the intracellular iron distribution associated with the uptake and trafficking of *H. pylori* through the cells[75], since *H. pylori* uptake by gastric cells is associated with an increase in total cellular iron content and its homeostasis depends on the transferrin receptor[76]. Indeed, a study by Flores *et al*[75] showed that *H. pylori* infection is associated with an increase in the total intracellular iron levels, redistribution of the transferrin receptor from the cell cytosol to the cell surface, and increased levels of ferritin. Moreover, Kato *et al*[77] showed that the SabA gene is highly expressed in bacterial isolates from iron deficient anemia patients, suggesting a role for this virulence factor in the development of anemia.

Non-alcoholic fatty liver disease: Non-alcoholic fatty acid disease (NAFLD) has also been suggested to be associated with *H. pylori* infection. In this context, a large number of reports, including cross-sectional studies, case reports and randomized-controlled studies have revealed a strong association between NAFLD and *H. pylori* infection[7]. In addition, it has been demonstrated in an animal model of *H. pylori* infection that the orally inoculated bacterium can reach the liver and cause hepatitis[78].

H. pylori infection may induce NAFLD by producing chronic systemic inflammation, increasing the levels of inflammatory cytokines, such as IL-6 and TNF-α, and activating NF-κB pathway, which induce insulin resistance (IR)[79]. The mechanisms of the pathogenesis of *H. pylori*-related inflammation in NAFLD involve directly reducing hepatocyte glycogen levels *via* a JNK signaling pathway[80], which in turn can down-regulate the expression of key genes involved in glucose metabolism and

accelerating lipolysis[81], thereby contributing indirectly to the development of IR. In addition, *H. pylori* may also induce white adipose tissue to release leptin, and then promote liver stearoyl- CoA desaturase, favoring the accumulation of fat deposits in the liver tissue[79].

Insulin resistance: Several studies have revealed a strong association between *H. pylori* and IR. In 2005, Aydemir *et al*[82] confirmed the existence of a positive correlation between chronic *H. pylori* infection and IR by showing that the homeostasis model assessment (HOMA-IR) of *H. pylori* positive subjects was significantly higher compared with *H. pylori* negative individuals. In another large cross-sectional study including 1,107 subjects, *H. pylori* seropositivity was significantly higher for patients with IR (HOMA-IR $\geq$ 2.5)[83]. Subsequent studies also confirmed the causal relationship between *H. pylori* and IR[84-86].

Mechanistically, *H. pylori*-induced IR may be caused indirectly by chronic inflammation or directly by activating certain signaling pathways. Several reports have confirmed that chronic inflammation is important for IR onset[87] and *H. pylori*-mediated chronic inflammation may increase the expression of C-reactive protein, TNF-α, and IL-6[88,89]. These cytokines activate IKK/NF-κB and JNK pathways, which may trigger IR by increasing insulin receptor phosphorylation on serine[81] or by inhibition of insulin receptor substrate-1 phosphorylation on tyrosine residues[90].

Despite such evidence, a recent systematic review revealed that *H. pylori* eradication does not improve insulin resistance, but may increase body weight (BW) and the body mass index (BMI), suggesting that further studies are needed to clarify the effect of *H. pylori* eradication on metabolism [8].

Type 2 diabetes mellitus: The association between Type 2 diabetes mellitus (T2DM) and *H. pylori* infection is controversial. Some studies indicate that the prevalence of *H. pylori* is higher in diabetic compared with non-diabetic patients[91,92], while others indicate that there are no differences between those groups[93]. Nevertheless, more recently, He *et al*[94] discussed the possibility that this controversy is likely due to inconsistencies in the methods used to define *H. pylori* positivity, diabetic status and the reduced sample sizes, among other limitations. More recently, a meta-analysis including 57397 individuals showed that there is significantly higher prevalence of *H. pylori* infection in diabetic type 2 patients as compared with healthy individuals[5].

The possible mechanisms linking *H. pylori* to diabetes include alterations in IR signaling, inflammation, accumulation of ROS and oxidative DNA damage in the gastric mucosa. It has been reported that ROS levels and oxidative DNA damage increase due to neutrophil infiltration in *H. pylori*-infected patients[95]. Moreover, a recent study performed in 100 patients showed increased serum levels of oxidative DNA damage (8-OHdG) and

oxidized low-density lipoprotein in T2DM patients positive for *H. pylori* infection[96].

Periodontitis: Periodontitis is characterized by the accumulation of bacterial plaque at the gingival margin, which induces an inflammatory response that leads to destruction of the connective tissue attachments to teeth, alveolar bone resorption and tooth loss[97]. The recent Global Burden of Disease Study (1990-2010) indicates that severe periodontitis is the 6th most prevalent disease worldwide, with an overall prevalence of 11.2%, although mild forms of this disease may reach over 90% of the population in developing countries[98]. Remarkably, periodontitis has also been linked to an increased risk in developing atherosclerosis, diabetes, rheumatoid arthritis and cancer[99-103]

Moreover, the oral cavity might represent a reservoir for *H. pylori*[104]. In a recent cross-sectional study that included 70 patients and 70 controls it was reported that the presence of *H. pylori* in dental plaque correlates with periodontitis and that the correlation appears to be better in severe forms of the disease [105]. Additionally, a study involving 40 patients (32 periodontitis and 8 controls) showed that periodontal disease positively correlates with gastric and oral *H. pylori* ($P < 0.005$)[106]. In this study, in spite of the small number of controls, it was shown that 70% of periodontitis patients have biopsies positive for *H. pylori*. Also, it is important to mention that 81% of periodontitis patients were positive for *H. pylori* in oral plaques.

Recently, Hu *et al*[107] demonstrated that *H. pylori* infected patients have worse periodontal parameters than non-infected individuals, suggesting that infection correlates the progression of the disease. This study also showed that the presence of periodontitis-associated bacteria was significantly higher in subjects with *H. pylori* infection than those without *H. pylori* infection. Moreover, the expression of inflammatory molecules, such as IL-8, IL-6 and IFN-γ significantly increased after *H. pylori* infection. Interestingly, those effects were associated with the presence of CagA[107].

ROLE OF INFLAMMATION IN *H. PYLORI* MEDIATED DISEASES

H. pylori infection triggers several adaptive cellular mechanisms in host cells that may favor gastric cancer development and progression[108]. However, whether the disease develops or not and the final outcome are thought to depend largely on the extent of inflammation promoted by the bacteria in the host during the pre-neoplastic process[109]. Indeed, chronic inflammation is a common causative event associated with the development of several types of cancer[110,111] and, as was demonstrated in animal models, *H. pylori* infection is considered the major factor responsible for gastric epithelial damage and deregulation of signaling leading to irreversible epigenetic changes in the gastric mucosa, a consistent hallmark

observed during the gastric carcinogenic cascade[19,109]. Initially, *H. pylori*-related studies focused on identifying the virulence factors implicated in these processes. Those factors epidemiologically associated with a higher risk of developing gastric cancer were tested *in vitro* and shown to induce signaling pathways associated with exacerbated inflammatory responses. For example, strains harboring particular *vacA* and *cagA* allele variants were found to induce elevated inflammatory responses in infected cells[112]. However, this seemingly simple scenario rapidly transited to a more complex one when epidemiological data revealed that in certain ubiquitously infected populations no correlation with elevated gastric cancer incidence was detected, as is the case for the so-called African enigma[113] where only a minor percentage of infected patients progress to develop cancer. To date, experimental studies have clarified that final disease outcome depends not only on the contribution of certain bacterial virulence factors, but also on host susceptibility, diet and environmental factors[114]. Here, it is important to mention that co-existence with the bacteria is not only to be viewed negatively (see previous and following chapters), bearing in mind that since the prevalence of the infection has declined in developed countries over the last decades, several disorders have emerged as a consequence of the lack of exposure to *H. pylori*[115]. Human beings have co-evolved with the bacterium and gastric as well as extra-gastric physiology has been conditioned to such association[116]. In order to persist in the gastric niche, *H. pylori* have evolved mechanisms necessary to evade and to attenuate the innate and adaptive immune systems by several mechanisms, including those implicated in evasion of recognition by pattern recognition receptors, inhibition of phagocytic killing, inhibition of killing by ROS and nitric oxide, among others[117]. Particularly, antigenic phase variation, modulation of adhesion molecules, immune inhibition by VacA protein and lipopolysaccharide (LPS) have been widely described[118]. Moreover, additional mitigating local mechanisms mediated by bacterial enzymes exist. For instance, it was recently reported that the expression of a cholesterol-α-glucosyltransferase reduced cholesterol levels in gastric epithelial cells, blocking IFN-γ signaling, a classical Th1 cytokine[119]. Notably, this enzyme is present in most *Helicobacter* species and cholesteryl α-glucosides are also involved in resistance to antibiotics[120], interference with phagosome trafficking[121], *H. pylori* type Ⅳ secretion system function[122] and immune evasion by inhibiting T-cell activation[123]. On the other hand, *H. pylori* superoxide dismutase (SOD) has been shown to suppress the production of pro-inflammatory cytokines during *in vivo* infection by reducing oxidative stress. Thus, SOD from *H. pylori* can inhibit the production of pro-inflammatory cytokines during *in vivo* infection[124]

Additionally, *H. pylori* deregulates adaptive immune responses by interfering with antigen presentation and modulation of T-cell responses[117,118]. Eradication of *H. pylori* has revealed the importance of this modulation of the immune response in preventing the development of extra-gastric immune and inflammatory disorders, such as gastroesophageal reflux disease, childhood asthma and allergy, as well as metabolic disorders[15,52,54]. Although in most of the cases correlations are derived from cross-sectional studies, the most experimentally validated preventive association is the appearance of childhood asthma[115,125]. Moreover, innate immune responses are also involved, given that bronchial epithelial cells, mast cells, basophils, natural killer T cells and dendritic cells (DC) also produce inflammatory mediators[52]. This harmful effector response is modulated by CD24+CD25+ regulatory cells (Treg) present in the lung, which secrete anti-inflammatory cytokines, such as IL-10 and transforming growth factor beta (TGF-β), preventing or modulating the Th2 responses to allergens[126]. Tregs of healthy individuals shift allergen-specific immune responses toward tolerance, thereby preventing the development of asthma and other allergic disorders[52]. Also in animal models of infection, the importance of dendritic cells in *H. pylori*-specific adaptive immune responses was noted. Particularly tolerance induction[52,127], Treg skewing and Th17 suppression observed in mice occurred in a *cagA*- and *vacA*-independent manner[128]. Chronic exposure to *H. pylori* impairs dendritic cell function and inhibits Th1 development[129]. Also, *H. pylori*-mediated protection was linked to IL-10-secretion by peripheral blood Treg cells[130].

The importance of these immune cells has been validated in experimental animal models of infection or induced asthma. For instance, blocking CD24+CD25+ Treg cells by a CD25-neutralizing antibody abrogated Treg cell tolerance promoted by *H. pylori* infection and enhanced pulmonary inflammation following albumin induced asthma[131]. Also, KO animals for IL-10, TGF-β or animals depleted of Tregs, develop gastritis to an elevated extent in response to *H. pylori* infection. Although these animals are able to clear the infection, pre-neoplastic lesions develop since Th1 responses predominate[52]. Together, these results indicate that tolerance rather than immunity protects against *H. pylori*-induced gastric pre-neoplastic lesions[132]. Accordingly, decreased Treg cell function is associated with an increase in peptic ulcer development upon *H. pylori* infection[133].

A currently unresolved question is how *H. pylori* promotes tolerance in distant organs such as the lung? A recent study showed that systemic and mucosal pre-administration of recombinant neutrophil-activating protein prevented ovalbumin-induced allergic asthma in mice, indicating that secreted virulence factors may be responsible[134]. What determines the balance between tolerance or elimination of an exacerbated Th1 type chronic inflammation could be explained in part by host hyperreactivity to allergens or bacterial infection. In this respect, pro-inflammatory cytokine polymorphisms are generally thought to participate in the genesis of gastric and other types of cancer[109] and interleukin family cytokines, like IL-1β and IL-18, have emerged as central mediators of mucosal inflammation[135]. On

the other hand, virulence factors can determine the type of response. For instance, blocking the TLR4 in a mouse model using a specific antibody prior to *H. pylori* infection, was shown to reduce the number of T-cell effectors (Th1 and Th17) and diminish the immune response[136]. In agreement with this observation, activation of TLR4 signaling was reported to be associated with gastric cancer progression by inducing mitochondrial ROS production[137]. Moreover, type I *H. pylori* (*cag* PAI+ and vacuolating toxin A+, VacA+) LPS exhibited a stimulatory effect on TLR4 signaling followed by mitogen oxidase 1 activation in cultured gastric pit cells through the lipid A portion of LPS[138]. In a similar study, LPS from some *H. pylori* strains were shown to act as TLR4 antagonists, which may contribute to more beneficial clinical outcomes of *H. pylori* infection in host individuals[139]. Additionally, *H. pylori* LPS from type I, but not type II strains, promotes cytotoxicity in cultured gastric mucosal cells[140]. Conversely, TLR2 mediates *H. pylori*-induced tolerogenic immune responses[141] and TLR9 signaling has anti-inflammatory effects during the early phase of *H. pylori*-induced gastritis in mice[142]. Also, additional virulence factors have been implicated in immune response suppression or tolerance, such as the suppression of dendritic cells by OipA *in vitro*[143], promotion of immune tolerance by VacA-mediated inhibition of T-cell proliferation and antigen-presentation[59]. Also, bacterial gamma-glutamyl transpeptidase[59] and outer membrane vesicles inhibit T-cell responses[144,145]. Furthermore, *H. pylori* infection has been associated inversely with IBD. Experimental immuno-regulatory properties of the *H. pylori* genome and particularly the immuno-regulatory sequence TTTAGGG was demonstrated to down-regulate dendritic cell-mediated production of pro-inflammatory cytokines both in an *in vitro* and *in vivo* model[146].

H. PYLORI COLONIZATION AND ITS ASSOCIATION WITH MICROBIOME SHIFTS

As discussed above, *H. pylori* infection has been associated both positively and negatively with the development of gastric and non-gastric diseases. Disease development is often linked to chronic inflammatory responses induced by *H. pylori*, as was discussed in the previous section. However, it is now becoming increasingly clear that *H. pylori* also induces changes in the host by altering the microbiome. This aspect will be covered in the following paragraphs.

The harsh gastric environment is thought to represent a key limitation to the complexity of the stomach microbiota[147]. This assumption, together with limitations imposed by culture-dependent strategies for bacterial identification, has historically leaded to an underestimation of the biodiversity in the stomach. In this context, the gastric microbiota was initially considered to include only a very select group of taxa,

including mainly *Veillonella* spp., *Lactobacillus* spp., and *Clostridium* spp., besides -of course- *H. pylori*[148-151]. Nonetheless, with the development of more sophisticated 16S rRNA-based bacterial identification techniques, we now have gleaned deeper insight to the complexity of the gastric microbiome. Accordingly, an increasing number of publications describe greater ecosystem diversity in the stomach and, importantly, correlate the presence of *H. pylori* with variations in the composition of the microbiome[23,48,152-156]. Bik *et al*[152] identified taxa, such as *Caulobacter*, *Actinobacillus*, *Corynebacterium*, *Rothia*, *Gemella*, *Leptotrichia*, *Porphyromonas*, *Capnocytophaga*, TM7, *Flexistipes*, and *Deinococcus* in the normal microbiome. Alternatively, Li *et al*[155] showed that the most common genera in gastric biopsies from both normal and non-*H. pylori* gastritis individuals were *Prevotella*, *Neisseria*, *Haemophilus*, and *Porphyromonas*. Later, Delgado *et al*[153] also identified *Propionibacterium*, *Lactobacillus* and *Streptococcus* as dominant genera in healthy samples.

Regarding the effect of *H. pylori* on the gastric microbiome, there is still some controversy. No effect on either diversity and/or evenness in community members between *H. pylori*-positive *vs H. pylori*-negative samples were observed at the phylum level[152]. Likewise, in a mouse model of *H. pylori* infection, neither acute nor chronic *H. pylori* infection altered the murine gastric microbiota[157]. Similarly, others described that, although when present *H. pylori* dominates the microbiome, only minor differences in community structure were observed in stomach biopsies from *H. pylori*-positive and negative subjects[158]. However, others have shown that the presence of *H. pylori* dramatically reduces the diversity of the gastric microbiota[20,21,23] and modifies the microbiome by increasing the relative abundance of *Proteobacteria*, *Spirochetes* and *Acidobacteria*, while decreasing *Actinobacteria*, *Bacteroidetes* and *Firmicutes*[156]. Similar results were reported by Thorell *et al*[30], who performed a meta-transcriptomic analysis and reported higher levels of *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* in subjects with low levels of *H. pylori*. Such discrepancies might be due to inter-subject variations, since the gastric microbiome seems to be sensitive to exogenous factors, such as diet and lifestyle, as has been shown by the analysis of monozygotic twins[159].

Interestingly, while the experimental inoculation of *H. pylori* into an established community in rhesus monkeys, did not affect the community membership or structure[160], pre-infection of mice with *H. pylori* did alter the microbiota structure in the stomach[22]. Therefore, the time-point in life when *H. pylori* is acquired is another aspect to be considered in the discrepancies reported for *H. pylori*-associated microbiome variations. Differences in both diversity and community composition were also observed in the stomachs of *H. pylori*-infected *vs H. pylori*-negative children and also in comparison to adults, regardless of the *H. pylori* status[161]. Thus, early acquisition of the bacterium is likely to shape the microbiome by inducing local modifications in the

stomach environment. One of these effects is driven by the production of ammonia and bicarbonate from urea[162,163]. Such compounds may serve as substrates for other bacteria[164], in addition to altering the stomach pH[162,163], which facilitates the colonization by other species, such as nitrogen-reducing bacteria[165]. Moreover, *H. pylori*-induced increases in the stomach pH favor the migration to the stomach of some bacterial taxa that are usually restricted to the intestinal tract (*Bacteroides* and *Clostridia*) in mice[166]. Interestingly, the effect of *H. pylori* on acid secretion depends on the pattern of gastritis that is induced[167]. In predominantly antral gastritis, the production of gastric acid is increased (hyperchlorhydria)[168], while in predominantly corpus gastritis, acid production decreases (hypochlorhydria)[169]. Thus, microbiome shifts may differ in both cases. In fact, hyperchlorhydria increases microbial diversity in the stomach[170] and it has been implicated in the development and progression of cancer (reviewed by Espinoza *et al* 2018[171]). Additionally, the viscosity of the gastric mucus layer decreases when the pH increases[172], making it easier for other microorganisms to colonize the epithelium. Finally, *H. pylori* can directly alter the mucus barrier by modulating the expression of stomach mucins[173].

As illustrated above, all these environmental modifications in the stomach may impact on the local microbiome, as well as induce changes in the entire gastrointestinal tract, since these are dynamic compartments between which fluids are exchanged and therefore microbes can easily migrate from one gastrointestinal segment to another[174]. Some of these *H. pylori*-mediated downstream effects in other compartments include impairment in the absorption of iron and vitamin B12 in the intestine[175,176], and alterations in carbohydrate and amino acid metabolism of the host[177]. Interestingly, besides the direct effect of *H. pylori* in the stomach/intestine, also the immune response triggered by the bacterium could affect the local microbiome, as well as bacterial populations at more distal sites in the human body.

Regarding the effect of *H. pylori*-mediated immune mediators in the microbiome, there are some contradictory reports. No statistically significant differences in the microbiota were found in CagA-positive (*n* = 10) compared to CagA-negative (*n* = 10) biopsies from human subjects[178]. Therefore, the increased production of pro-inflammatory cytokines mediated by CagA appears not to have an effect on the microbiome in this model. Nonetheless, as the sample cohort was small in that study, this question probably needs to be re-evaluated in larger groups of samples. In contrast, in a transgenic *Drosophila* model of CagA expression, CagA was sufficient to alter midgut host microbiota[179]. Additionally, a series of reports demonstrated differences in the intestinal microbiome related to the presence of *H. pylori* both in humans[180] and mice[22]. Moreover Schulz *et al*[158] correlated the presence of *H. pylori* in human individuals with modifications in the microbiome of the duodenum and the oral cavity. More specifically,

Heimesaat *et al*[181] demonstrated that chronic infection of Mongolian gerbils with *H. pylori* resulted in changes in some specific genera, including increased abundance in the large intestine of *Akkermansia*, which is involved in mucus degradation. These changes were accompanied by variations in the expression of immunity-related genes in both the stomach and the lung, with stronger effects in the former. The authors speculated that early community shifts could reflect changes in the niche microenvironment (*e.g.*, altered gastric pH), while later shifts might be driven by the cumulative changes in the immune/inflammatory response triggered by *H. pylori*. These effects could also be observed at distant sites in the host organism and be driven by other members of the *Helicobacter* genus. For instance, natural colonization of the mouse digestive tract with *Helicobacter hepaticus* leads to a shift in gut microbiota, which generates subclinical inflammation and a drastic impairment of the control of *Mycobacterium tuberculosis* growth by the immune system[182].

It is likely that *H. pylori* might affect mucosal diseases at distant sites *via* its effects on immune cells that traffic through lymphatic vessels. *H. pylori* also induces a shift in the immune response toward an induction of Treg cells, mediated by VacA and GGT[59,161,183,184]. Treg cell responses are important in differentiating between self and foreign antigens, *i.e.*, immunological tolerance. This effect could be involved in *H. pylori* persistence in the stomach, and also could contribute to suppressing gastric inflammation, which may explain reduced gastric disease severity in *H. pylori*-positive children compared to *H. pylori*-positive adults[161]. Such effects have been linked to alterations in the stomach physiology and its microbiota, as well as to progression of extra-gastric diseases, such as asthma[59], celiac disease[17], ischemic heart diseases, insulin resistance, Type 2 diabetes mellitus, periodontal diseases, among others (see previous sections and references listed in Table 1).

Interestingly, as was stated above, *H. pylori* is part of a complex microbiota in the stomach and its presence has been linked to modifications in the microbiome at other sites in the body. Therefore, it is reasonable to speculate that other community members could also contribute to changes observed following *H. pylori* infection of the host. Indeed, direct bacteria-bacteria interaction was described by Khosravi *et al*[185] between *H. pylori* and *Streptococcus mitis,* in which *H. pylori* transit from spiral to coccoid-shaped cells that are more resistant to stressing conditions. The exact effect of this conversion on disease progression remains to be elucidated. Also, antimicrobial molecules produced by *Lactobacillus spp* have been shown to be active against *H. pylori* strains[186-189]. Therefore, bidirectional communication and modulation between *H. pylori* and other community members occur, and the combination of all these interactions will be reflected in the host health status. The question as to how the microbiota shapes the immune system and how this affects its response to some diseases has been broadly discussed in the literature (see for instance,

Hooper *et al*[190] 2012). Therefore, modifications in the microbiome induced by early acquisition of *H. pylori*[161] may also determine the host immune status and, as a consequence, the development of a number of systemic diseases. This is relevant, since Rolig *et al*[191] reported that when the microbiota is altered by antibiotic treatment, the *H. pylori*-triggered inflammation is reduced.

CONCLUSION

In summary, *H. pylori* has a strong effect in the stomach microenvironment and also on the host immunological status, leading to shifts in the microbiome at different sites of the body. These shifts are involved not only in the pathogenesis of gastric diseases, but also in some of the non-gastric *H. pylori*-related diseases that were discussed in this review. The question as to whether the bacterium is acquired early on or later in life is a key point when analyzing such effects, as *H. pylori* has been reported to co-evolve with its host, shaping the immune system and consequently, the microbiome. Therefore, a better understanding of the exact mechanisms involved in such effects, as well as the *H. pylori-induced* microbiome shifts that may be related to the development of specific diseases, will likely be useful to predict and hopefully prevent *H. pylori*-associated diseases. Ideally, one would aspire to achieving this goal while at the same time preserving the beneficial effects that *H. pylori*-host cohabitation appears also to offer.

REFERENCES

1   **de Martel C**, Ferlay J, Franceschi S, Vignat J, Bray F, Forman D, Plummer M. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet Oncol* 2012; **13**: 607-615 [PMID: 22575588 DOI: 10.1016/S1470-2045(12)70137-7]

2   **Suerbaum S**, Michetti P. Helicobacter pylori infection. *N Engl J Med* 2002; **347**: 1175-1186 [PMID: 12374879 DOI: 10.1056/NEJMra020542]

3   **Shmuely H**, Wattad M, Solodky A, Yahav J, Samra Z, Zafrir N. Association of Helicobacter pylori with coronary artery disease and myocardial infarction assessed by myocardial perfusion imaging. *Isr Med Assoc J* 2014; **16**: 341-346 [PMID: 25058994]

4   **Liu J**, Wang F, Shi S. Helicobacter pylori Infection Increase the Risk of Myocardial Infarction: A Meta-Analysis of 26 Studies Involving more than 20,000 Participants. *Helicobacter* 2015; **20**: 176-183 [PMID: 25382293 DOI: 10.1111/hel.12188]

5   **Li JZ**, Li JY, Wu TF, Xu JH, Huang CZ, Cheng D, Chen QK, Yu T. Helicobacter pylori Infection Is Associated with Type 2 Diabetes, Not Type 1 Diabetes: An Updated Meta-Analysis. *Gastroenterol Res Pract* 2017; **2017**: 5715403 [PMID: 28883831 DOI: 10.1155/2017/5715403]

6   **Xu MY**, Cao B, Yuan BS, Yin J, Liu L, Lu QB. Association of anaemia with Helicobacter pylori infection: a retrospective study. *Sci Rep* 2017; **7**: 13434 [PMID: 29044219 DOI: 10.1038/s41598-017-13955-3]

7   **Chen CX**, Mao YS, Foster P, Zhu ZW, Du J, Guo CY. Possible association between Helicobacter pylori infection and nonalcoholic fatty liver disease. *Appl Physiol Nutr Metab* 2017; **42**: 295-301 [PMID: 28177748 DOI: 10.1139/apnm-2016-0499]

8   **Upala S**, Sanguankeo A, Saleem SA, Jaruvongvanich V. Effects of Helicobacter pylori eradication on insulin resistance and metabolic parameters: a systematic review and meta-analysis. *Eur J Gastroenterol Hepatol* 2017; **29**: 153-159 [PMID: 27832037 DOI:

10.1097/MEG.0000000000000774]

9   **Bach JF**. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 2002; **347**: 911-920 [PMID: 12239261 DOI: 10.1056/NEJMra020100]

10   **Chen Y**, Blaser MJ. Inverse associations of Helicobacter pylori with asthma and allergy. *Arch Intern Med* 2007; **167**: 821-827 [PMID: 17452546 DOI: 10.1001/archinte.167.8.821]

11   **Cohen D**, Shoham O, Orr N, Muhsen K. An inverse and independent association between Helicobacter pylori infection and the incidence of shigellosis and other diarrheal diseases. *Clin Infect Dis* 2012; **54**: e35-e42 [PMID: 22157171 DOI: 10.1093/cid/cir916]

12   **Islami F**, Kamangar F. Helicobacter pylori and esophageal cancer risk: a meta-analysis. *Cancer Prev Res* (Phila) 2008; **1**: 329-338 [PMID: 19138977 DOI: 10.1158/1940-6207.CAPR-08-0109]

13   **Perry S**, de Jong BC, Solnick JV, de la Luz Sanchez M, Yang S, Lin PL, Hansen LM, Talat N, Hill PC, Hussain R, Adegbola RA, Flynn J, Canfield D, Parsonnet J. Infection with Helicobacter pylori is associated with protection against tuberculosis. *PLoS One* 2010; **5**: e8804 [PMID: 20098711 DOI: 10.1371/journal.pone.0008804]

14   **Sonnenberg A**, Dellon ES, Turner KO, Genta RM. The influence of Helicobacter pylori on the ethnic distribution of esophageal eosinophilia. *Helicobacter* 2017; **22**: e12370 [PMID: 28029200 DOI: 10.1111/hel.12370]

15   **Miftahussurur M**, Nusi IA, Graham DY, Yamaoka Y. Helicobacter, Hygiene, Atopy, and Asthma. *Front Microbiol* 2017; **8**: 1034 [PMID: 28642748 DOI: 10.3389/fmicb.2017.01034]

16   **Efthymiou G**, Dardiotis E, Liaskos C, Marou E, Tsimourtou V, Scheper T, Meyer W, Daponte A, Sakkas LI, Hadjigeorgiou G, Bogdanos DP. Anti-hsp60 antibody responses based on Helicobacter pylori in patients with multiple sclerosis: (ir)Relevance to disease pathogenesis. *J Neuroimmunol* 2016; **298**: 19-23 [PMID: 27609271 DOI: 10.1016/j.jneuroim.2016.06.009]

17   **Lucero Y**, Oyarzún A, O'Ryan M, Quera R, Espinosa N, Valenzuela R, Simian D, Alcalde E, Arce C, Farfán MJ, Vergara AF, Gajardo I, Mendez J, Carrasco J, Errázuriz G, Gonzalez M, Ossa JC, Maiza E, Perez-Bravo F, Castro M, Araya M. Helicobacter pylori cagA+ Is Associated with Milder Duodenal Histological Changes in Chilean Celiac Patients. *Front Cell Infect Microbiol* 2017; **7**: 376 [PMID: 28879170 DOI: 10.3389/fcimb.2017.00376]

18   **Narang M**, Puri AS, Sachdeva S, Singh J, Kumar A, Saran RK. Celiac disease and Helicobacter pylori infection in children: Is there any Association? *J Gastroenterol Hepatol* 2017; **32**: 1178-1182 [PMID: 27862319 DOI: 10.1111/jgh.13654]

19   **Niwa T**, Toyoda T, Tsukamoto T, Mori A, Tatematsu M, Ushijima T. Prevention of Helicobacter pylori-induced gastric cancers in gerbils by a DNA demethylating agent. *Cancer Prev Res (Phila)* 2013; **6**: 263-270 [PMID: 23559452 DOI: 10.1158/1940-6207.CAPR-12-0369]

20   **Andersson AF**, Lindberg M, Jakobsson H, Bäckhed F, Nyrén P, Engstrand L. Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PLoS One* 2008; **3**: e2836 [PMID: 18665274 DOI: 10.1371/journal.pone.0002836]

21   **Das A**, Pereira V, Saxena S, Ghosh TS, Anbumani D, Bag S, Das B, Nair GB, Abraham P, Mande SS. Gastric microbiome of Indian patients with Helicobacter pylori infection, and their interaction networks. *Sci Rep* 2017; **7**: 15438 [PMID: 29133866 DOI: 10.1038/s41598-017-15510-6]

22   **Kienesberger S**, Cox LM, Livanos A, Zhang XS, Chung J, Perez-Perez GI, Gorkiewicz G, Zechner EL, Blaser MJ. Gastric Helicobacter pylori Infection Affects Local and Distant Microbial Populations and Host Responses. *Cell Rep* 2016; **14**: 1395-1407 [PMID: 26854236 DOI: 10.1016/j.celrep.2016.01.017]

23   **Llorca L**, Pérez-Pérez G, Urruzuno P, Martinez MJ, Iizumi T, Gao Z, Sohn J, Chung J, Cox L, Simón-Soro A, Mira A, Alarcón T. Characterization of the Gastric Microbiota in a Pediatric Population According to Helicobacter pylori Status. *Pediatr Infect Dis J* 2017; **36**: 173-178 [PMID: 27820723 DOI: 10.1097/INF.0000000000001383]

24   **Plummer M**, de Martel C, Vignat J, Ferlay J, Bray F, Franceschi S. Global burden of cancers attributable to infections in 2012: a synthetic analysis. *Lancet Glob Health* 2016; **4**: e609-e616 [PMID:

27470177 DOI: 10.1016/S2214-109X(16)30143-7]

25 **Linz B**, Balloux F, Moodley Y, Manica A, Liu H, Roumagnac P, Falush D, Stamer C, Prugnolle F, van der Merwe SW, Yamaoka Y, Graham DY, Perez-Trallero E, Wadstrom T, Suerbaum S, Achtman M. An African origin for the intimate association between humans and Helicobacter pylori. *Nature* 2007; **445**: 915-918 [PMID: 17287725 DOI: 10.1038/nature05562]

26 **Achtman M**, Azuma T, Berg DE, Ito Y, Morelli G, Pan ZJ, Suerbaum S, Thompson SA, van der Ende A, van Doorn LJ. Recombination and clonal groupings within Helicobacter pylori from different geographical regions. *Mol Microbiol* 1999; **32**: 459-470 [PMID: 10320570 DOI: 10.1046/j.1365-2958.1999.01382.x]

27 **Morelli G**, Didelot X, Kusecek B, Schwarz S, Bahlawane C, Falush D, Suerbaum S, Achtman M. Microevolution of Helicobacter pylori during prolonged infection of single hosts and within families. *PLoS Genet* 2010; **6**: e1001036 [PMID: 20661309 DOI: 10.1371/journal.pgen.1001036]

28 **Schwarz S**, Morelli G, Kusecek B, Manica A, Balloux F, Owen RJ, Graham DY, van der Merwe S, Achtman M, Suerbaum S. Horizontal versus familial transmission of Helicobacter pylori. *PLoS Pathog* 2008; **4**: e1000180 [PMID: 18949030 DOI: 10.1371/journal.ppat.1000180]

29 **Falush D**, Wirth T, Linz B, Pritchard JK, Stephens M, Kidd M, Blaser MJ, Graham DY, Vacher S, Perez-Perez GI, Yamaoka Y, Mégraud F, Otto K, Reichard U, Katzowitsch E, Wang X, Achtman M, Suerbaum S. Traces of human migrations in Helicobacter pylori populations. *Science* 2003; **299**: 1582-1585 [PMID: 12624269 DOI: 10.1126/science.1080857]

30 **Thorell K**, Yahara K, Berthenet E, Lawson DJ, Mikhail J, Kato I, Mendez A, Rizzato C, Bravo MM, Suzuki R, Yamaoka Y, Torres J, Sheppard SK, Falush D. Correction: Rapid evolution of distinct Helicobacter pylori subpopulations in the Americas. *PLoS Genet* 2017; **13**: e1006730 [PMID: 28410409 DOI: 10.1371/journal.pgen.1006730]

31 **Agnew P**, Koella JC. Virulence, parasite mode of transmission, and host fluctuating asymmetry. *Proc Biol Sci* 1997; **264**: 9-15 [PMID: 9061958 DOI: 10.1098/rspb.1997.0002]

32 **Ahmed N**, Tenguria S, Nandanwar N. Helicobacter pylori--a seasoned pathogen by any other name. *Gut Pathog* 2009; **1**: 24 [PMID: 20030808 DOI: 10.1186/1757-4749-1-24]

33 **Hatakeyama M**. [Oncogenic mechanism of Helicobacter pylori]. *Nihon Rinsho Meneki Gakkai Kaishi* 2008; **31**: 132-140 [PMID: 18587223 DOI: 10.2177/jsci.31.132]

34 **Atherton JC**, Tham KT, Peek RM Jr, Cover TL, Blaser MJ. Density of Helicobacter pylori infection in vivo as assessed by quantitative culture and histology. *J Infect Dis* 1996; **174**: 552-556 [PMID: 8769613 DOI: 10.1093/infdis/174.3.552]

35 **Devi SM**, Ahmed I, Khan AA, Rahman SA, Alvi A, Sechi LA, Ahmed N. Genomes of Helicobacter pylori from native Peruvians suggest admixture of ancestral and modern lineages and reveal a western type cag-pathogenicity island. *BMC Genomics* 2006; **7**: 191 [PMID: 16872520 DOI: 10.1186/1471-2164-7-191]

36 **Kodaman N**, Pazos A, Schneider BG, Piazuelo MB, Mera R, Sobota RS, Sicinschi LA, Shaffer CL, Romero-Gallo J, de Sablet T, Harder RH, Bravo LE, Peek RM Jr, Wilson KT, Cover TL, Williams SM, Correa P. Human and Helicobacter pylori coevolution shapes the risk of gastric disease. *Proc Natl Acad Sci USA* 2014; **111**: 1455-1460 [PMID: 24474772 DOI: 10.1073/pnas.1318093111]

37 **Gutiérrez-Escobar AJ**, Trujillo E, Acevedo O, Bravo MM. Phylogenomics of Colombian Helicobacter pylori isolates. *Gut Pathog* 2017; **9**: 52 [PMID: 28912838 DOI: 10.1186/s13099-017-0201-1]

38 **Duncan SS**, Valk PL, McClain MS, Shaffer CL, Metcalf JA, Bordenstein SR, Cover TL. Comparative genomic analysis of East Asian and non-Asian Helicobacter pylori strains identifies rapidly evolving genes. *PLoS One* 2013; **8**: e55120 [PMID: 23383074 DOI: 10.1371/journal.pone.0055120]

39 **Kauser F**, Khan AA, Hussain MA, Carroll IM, Ahmad N, Tiwari S, Shouche Y, Das B, Alam M, Ali SM, Habibullah CM, Sierra R, Megraud F, Sechi LA, Ahmed N. The cag pathogenicity island of Helicobacter pylori is disrupted in the majority of patient isolates from different human populations. *J Clin Microbiol* 2004; **42**: 5302-5308 [PMID: 15528729 DOI: 10.1128/JCM.42.11.5302-5308.2004]

40 **Mhaskar RS**, Ricardo I, Azliyati A, Laxminarayan R, Amol B, Santosh W, Boo K. Assessment of risk factors of helicobacter pylori infection and peptic ulcer disease. *J Glob Infect Dis* 2013; **5**: 60-67 [PMID: 23853433 DOI: 10.4103/0974-777X.112288]

41 **El-Omar EM**, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF Jr, Rabkin CS. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* 2000; **404**: 398-402 [PMID: 10746728 DOI: 10.1038/35006081]

42 **El-Omar EM**, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF Jr, Rabkin CS. The role of interleukin-1 polymorphisms in the pathogenesis of gastric cancer. *Nature* 2001; **412**: 99 [PMID: 11808612 DOI: 10.1038/35083631]

43 **El-Omar EM**, Rabkin CS, Gammon MD, Vaughan TL, Risch HA, Schoenberg JB, Stanford JL, Mayne ST, Goedert J, Blot WJ, Fraumeni JF Jr, Chow WH. Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms. *Gastroenterology* 2003; **124**: 1193-1201 [PMID: 12730860 DOI: 10.1016/S0016-5085(03)00157-4]

44 **Figueiredo C**, Machado JC, Pharoah P, Seruca R, Sousa S, Carvalho R, Capelinha AF, Quint W, Caldas C, van Doorn LJ, Carneiro F, Sobrinho-Simões M. Helicobacter pylori and interleukin 1 genotyping: an opportunity to identify high-risk individuals for gastric carcinoma. *J Natl Cancer Inst* 2002; **94**: 1680-1687 [PMID: 12441323 DOI: 10.1093/jnci/94.22.1680]

45 **Garza-González E**, Bosques-Padilla FJ, El-Omar E, Hold G, Tijerina-Menchaca R, Maldonado-Garza HJ, Pérez-Pérez GI. Role of the polymorphic IL-1B, IL-1RN and TNF-A genes in distal gastric cancer in Mexico. *Int J Cancer* 2005; **114**: 237-241 [PMID: 15540224 DOI: 10.1002/ijc.20718]

46 **Ando T**, Yoshida T, Enomoto S, Asada K, Tatematsu M, Ichinose M, Sugiyama T, Ushijima T. DNA methylation of microRNA genes in gastric mucosae of gastric cancer patients: its possible involvement in the formation of epigenetic field defect. *Int J Cancer* 2009; **124**: 2367-2374 [PMID: 19165869 DOI: 10.1002/ijc.24219]

47 **Shin CM**, Kim N, Jung Y, Park JH, Kang GH, Kim JS, Jung HC, Song IS. Role of Helicobacter pylori infection in aberrant DNA methylation along multistep gastric carcinogenesis. *Cancer Sci* 2010; **101**: 1337-1346 [PMID: 20345486 DOI: 10.1111/j.1349-7006.2010.01535.x]

48 **Monstein HJ**, Tiveljung A, Kraft CH, Borch K, Jonasson J. Profiling of bacterial flora in gastric biopsies from patients with Helicobacter pylori-associated gastritis and histologically normal control individuals by temperature gradient gel electrophoresis and 16S rDNA sequence analysis. *J Med Microbiol* 2000; **49**: 817-822 [PMID: 10966230 DOI: 10.1099/0022-1317-49-9-817]

49 **Song Q**, Lange T, Spahr A, Adler G, Bode G. Characteristic distribution pattern of Helicobacter pylori in dental plaque and saliva detected with nested PCR. *J Med Microbiol* 2000; **49**: 349-353 [PMID: 10755629 DOI: 10.1099/0022-1317-49-4-349]

50 **Pest P**, Zárate J, Varsky C, Man F, Schraier M. Helicobacter pylori in recently-diagnosed versus chronic duodenal ulcer. *Acta Gastroenterol Latinoam* 1996; **26**: 273-276 [PMID: 9363263]

51 **Mishra S**. Is Helicobacter pylori good or bad? *Eur J Clin Microbiol Infect Dis* 2013; **32**: 301-304 [PMID: 23132690 DOI: 10.1007/s10096-012-1773-9]

52 **Arnold IC**, Hitzler I, Müller A. The immunomodulatory properties of Helicobacter pylori confer protection against allergic and chronic inflammatory disorders. *Front Cell Infect Microbiol* 2012; **2**: 10 [PMID: 22919602 DOI: 10.3389/fcimb.2012.00010]

53 **Holster IL**, Vila AM, Caudri D, den Hoed CM, Perez-Perez GI, Blaser MJ, de Jongste JC, Kuipers EJ. The impact of Helicobacter pylori on atopic disorders in childhood. *Helicobacter* 2012; **17**: 232-237 [PMID: 22515362 DOI: 10.1111/j.1523-5378.2012.00934.x]

54 **den Hollander WJ**, Sonnenschein-van der Voort AM, Holster IL, de Jongste JC, Jaddoe VW, Hofman A, Perez-Perez GI, Moll HA, Blaser MJ, Duijts L, Kuipers EJ. Helicobacter pylori in children with asthmatic conditions at school age, and their mothers. *Aliment Pharmacol Ther* 2016; **43**: 933-943 [PMID: 26932510 DOI: 10.1111/apt.13572]

55 **Chen Y**, Blaser MJ. Helicobacter pylori colonization is inversely associated with childhood asthma. *J Infect Dis* 2008; **198**: 553-560 [PMID: 18598192 DOI: 10.1086/590158]

56 **Bamford KB**, Fan X, Crowe SE, Leary JF, Gourley WK, Luthra GK, Brooks EG, Graham DY, Reyes VE, Ernst PB. Lymphocytes in the human gastric mucosa during Helicobacter pylori have a T helper cell 1 phenotype. *Gastroenterology* 1998; **114**: 482-492 [PMID: 9496938 DOI: 10.1016/S0016-5085(98)70531-1]

57 **Sommer F**, Faller G, Konturek P, Kirchner T, Hahn EG, Zeus J, Röllinghoff M, Lohoff M. Antrum- and corpus mucosa-infiltrating CD4(+) lymphocytes in Helicobacter pylori gastritis display a Th1 phenotype. *Infect Immun* 1998; **66**: 5543-5546 [PMID: 9784570]

58 **Lundgren A**, Trollmo C, Edebo A, Svennerholm AM, Lundin BS. Helicobacter pylori-specific CD4+ T cells home to and accumulate in the human Helicobacter pylori-infected gastric mucosa. *Infect Immun* 2005; **73**: 5612-5619 [PMID: 16113278 DOI: 10.1128/IAI.73.9.5612-5619.2005]

59 **Oertli M**, Noben M, Engler DB, Semper RP, Reuter S, Maxeiner J, Gerhard M, Taube C, Müller A. Helicobacter pylori γ-glutamyl transpeptidase and vacuolating cytotoxin promote gastric persistence and immune tolerance. *Proc Natl Acad Sci USA* 2013; **110**: 3047-3052 [PMID: 23382221 DOI: 10.1073/pnas.1211248110]

60 **Dela Pena-Ponce MG**, Jimenez MT, Hansen LM, Solnick JV, Miller LA. The Helicobacter pylori type IV secretion system promotes IL-8 synthesis in a model of pediatric airway epithelium via p38 MAP kinase. *PLoS One* 2017; **12**: e0183324 [PMID: 28813514 DOI: 10.1371/journal.pone.0183324]

61 **Higgins PD**, Johnson LA, Luther J, Zhang M, Sauder KL, Blanco LP, Kao JY. Prior Helicobacter pylori infection ameliorates Salmonella typhimurium-induced colitis: mucosal crosstalk between stomach and distal intestine. *Inflamm Bowel Dis* 2011; **17**: 1398-1408 [PMID: 21560200 DOI: 10.1002/ibd.21489]

62 **Lord AR**, Simms LA, Hanigan K, Sullivan R, Hobson P, Radford-Smith GL. Protective effects of Helicobacter pylori for IBD are related to the cagA-positive strain. *Gut* 2018; **67**: 393-394 [PMID: 28408384 DOI: 10.1136/gutjnl-2017-313805]

63 **Castaño-Rodríguez N**, Kaakoush NO, Lee WS, Mitchell HM. Dual role of Helicobacter and Campylobacter species in IBD: a systematic review and meta-analysis. *Gut* 2017; **66**: 235-249 [PMID: 26508508 DOI: 10.1136/gutjnl-2015-310545]

64 **Rubio-Tapia A**, Kyle RA, Kaplan EL, Johnson DR, Page W, Erdtmann F, Brantner TL, Kim WR, Phelps TK, Lahr BD, Zinsmeister AR, Melton LJ 3rd, Murray JA. Increased prevalence and mortality in undiagnosed celiac disease. *Gastroenterology* 2009; **137**: 88-93 [PMID: 19362553 DOI: 10.1053/j.gastro.2009.03.059]

65 **Lebwohl B**, Blaser MJ, Ludvigsson JF, Green PH, Rundle A, Sonnenberg A, Genta RM. Decreased risk of celiac disease in patients with Helicobacter pylori colonization. *Am J Epidemiol* 2013; **178**: 1721-1730 [PMID: 24124196 DOI: 10.1093/aje/kwt234]

66 **Grigoriadis N**, van Pesch V; ParadigMS Group. A basic overview of multiple sclerosis immunopathology. *Eur J Neurol* 2015; **22** Suppl 2: 3-13 [PMID: 26374508 DOI: 10.1111/ene.12798]

67 **Yao G**, Wang P, Luo XD, Yu TM, Harris RA, Zhang XM. Meta-analysis of association between Helicobacter pylori infection and multiple sclerosis. *Neurosci Lett* 2016; **620**: 1-7 [PMID: 27033666 DOI: 10.1016/j.neulet.2016.03.037]

68 **Jaruvongvanich V**, Sanguankeo A, Jaruvongvanich S, Upala S. Association between Helicobacter pylori infection and multiple sclerosis: A systematic review and meta-analysis. *Mult Scler Relat Disord* 2016; **7**: 92-97 [PMID: 27237767 DOI: 10.1016/j.msard.2016.03.013]

69 **Alvarez-Arellano L**, Maldonado-Bernal C. Helicobacter pylori and neurological diseases: Married by the laws of inflammation. *World J Gastrointest Pathophysiol* 2014; **5**: 400-404 [PMID: 25400983 DOI: 10.4291/wjgp.v5.i4.400]

70 **Boziki M**, Polyzos SA, Deretzi G, Kazakos E, Katsinelos P, Doulberis M, Kotronis G, Giartza-Taxidou E, Laskaridis L, Tzivras D, Vardaka E, Kountouras C, Grigoriadis N, Thomann R, Kountouras J. A potential impact of Helicobacter pylori-related galectin-3 in neurodegeneration. *Neurochem Int* 2018; **113**: 137-151 [PMID: 29246761 DOI: 10.1016/j.neuint.2017.12.003]

71 **Testerman TL**, Morris J. Beyond the stomach: an updated view of Helicobacter pylori pathogenesis, diagnosis, and treatment. *World J Gastroenterol* 2014; **20**: 12781-12808 [PMID: 25278678 DOI: 10.3748/wjg.v20.i36.12781]

72 **Huang WS**, Tseng CH, Lin CL, Tsai CH, Kao CH. Helicobacter pylori infection increases subsequent ischemic stroke risk: a nationwide population-based retrospective cohort study. *QJM* 2014; **107**: 969-975 [PMID: 24890556 DOI: 10.1093/qjmed/hcu117]

73 **Sharma V**, Aggarwal A. Helicobacter pylori: Does it add to risk of coronary artery disease. *World J Cardiol* 2015; **7**: 19-25 [PMID: 25632315 DOI: 10.4330/wjc.v7.i1.19]

74 **Konno M**, Muraoka S, Takahashi M, Imai T. Iron-deficiency anemia associated with Helicobacter pylori gastritis. *J Pediatr Gastroenterol Nutr* 2000; **31**: 52-56 [PMID: 10896071 DOI: 10.1097/00005176-200007000-00012]

75 **Flores SE**, Aitchison A, Day AS, Keenan JI. Helicobacter pylori infection perturbs iron homeostasis in gastric epithelial cells. *PLoS One* 2017; **12**: e0184026 [PMID: 28873091 DOI: 10.1371/journal.pone.0184026]

76 **Flores SE**, Day AS, Keenan JI. Measurement of total iron in Helicobacter pylori-infected gastric epithelial cells. *Biometals* 2015; **28**: 143-150 [PMID: 25428685 DOI: 10.1007/s10534-014-9810-z]

77 **Kato S**, Osaki T, Kamiya S, Zhang XS, Blaser MJ. Helicobacter pylori sabA gene is associated with iron deficiency anemia in childhood and adolescence. *PLoS One* 2017; **12**: e0184046 [PMID: 28854239 DOI: 10.1371/journal.pone.0184046]

78 **Huang Y**, Tian XF, Fan XG, Fu CY, Zhu C. The pathological effect of Helicobacter pylori infection on liver tissues in mice. *Clin Microbiol Infect* 2009; **15**: 843-849 [PMID: 19392901 DOI: 10.1111/j.1469-0691.2009.02719.x]

79 **Cheng DD**, He C, Ai HH, Huang Y, Lu NH. The Possible Role of Helicobacter pylori Infection in Non-alcoholic Fatty Liver Disease. *Front Microbiol* 2017; **8**: 743 [PMID: 28539915 DOI: 10.3389/fmicb.2017.00743]

80 **Li L**, He Q, Huang X, Man Y, Zhou Y, Wang S, Wang J, Li J. NOX3-derived reactive oxygen species promote TNF-alpha-induced reductions in hepatocyte glycogen levels via a JNK pathway. *FEBS Lett* 2010; **584**: 995-1000 [PMID: 20102709 DOI: 10.1016/j.febslet.2010.01.044]

81 **Hotamisligil GS**, Johnson RS, Distel RJ, Ellis R, Papaioannou VE, Spiegelman BM. Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. *Science* 1996; **274**: 1377-1379 [PMID: 8910278 DOI: 10.1126/science.274.5291.1377]

82 **Aydemir S**, Bayraktaroglu T, Sert M, Sokmen C, Atmaca H, Mungan G, Gun BD, Borazan A, Ustundag Y. The effect of Helicobacter pylori on insulin resistance. *Dig Dis Sci* 2005; **50**: 2090-2093 [PMID: 16240220 DOI: 10.1007/s10620-005-3012-z]

83 **Gunji T**, Matsuhashi N, Sato H, Fujibayashi K, Okumura M, Sasabe N, Urabe A. Helicobacter pylori infection significantly increases insulin resistance in the asymptomatic Japanese population. *Helicobacter* 2009; **14**: 144-150 [PMID: 19751440 DOI: 10.1111/j.1523-5378.2009.00705.x]

84 **Chen LW**, Chien CY, Yang KJ, Kuo SF, Chen CH, Chien RN. Helicobacter pylori Infection Increases Insulin Resistance and Metabolic Syndrome in Residents Younger than 50 Years Old: A Community-Based Study. *PLoS One* 2015; **10**: e0128671 [PMID: 26020514 DOI: 10.1371/journal.pone.0128671]

85 **Polyzos SA**, Kountouras J, Zavos C, Deretzi G. Helicobacter pylori Infection and insulin resistance. *Helicobacter* 2013; **18**: 165-166 [PMID: 23066701 DOI: 10.1111/hel.12019]

86 **Polyzos SA**, Kountouras J, Zavos C, Deretzi G. The association between Helicobacter pylori infection and insulin resistance: a

systematic review. *Helicobacter* 2011; **16**: 79-88 [PMID: 21435084 DOI: 10.1111/j.1523-5378.2011.00822.x]

87   **Hossain IA**, Akter S, Bhuiyan FR, Shah MR, Rahman MK, Ali L. Subclinical inflammation in relation to insulin resistance in prediabetic subjects with nonalcoholic fatty liver disease. *BMC Res Notes* 2016; **9**: 266 [PMID: 27169860 DOI: 10.1186/s13104-016-2071-x]

88   **Tsai S**, Clemente-Casares X, Revelo XS, Winer S, Winer DA. Are obesity-related insulin resistance and type 2 diabetes autoimmune diseases? *Diabetes* 2015; **64**: 1886-1897 [PMID: 25999531 DOI: 10.2337/db14-1488]

89   **Yildirim Z**, Bozkurt B, Ozol D, Armutcu F, Akgedik R, Karamanli H, Kizilirmak D, İkizek M. Increased Exhaled 8-Isoprostane and Interleukin-6 in Patients with Helicobacter pylori Infection. *Helicobacter* 2016; **21**: 389-394 [PMID: 27061444 DOI: 10.1111/hel.12302]

90   **Dandona P**, Aljada A, Bandyopadhyay A. Inflammation: the link between insulin resistance, obesity and diabetes. *Trends Immunol* 2004; **25**: 4-7 [PMID: 14698276 DOI: 10.1016/j.it.2003.10.013]

91   **Bener A**, Micallef R, Afifi M, Derbala M, Al-Mulla HM, Usmani MA. Association between type 2 diabetes mellitus and Helicobacter pylori infection. *Turk J Gastroenterol* 2007; **18**: 225-229 [PMID: 18080918]

92   **Devrajani BR**, Shah SZ, Soomro AA, Devrajani T. Type 2 diabetes mellitus: A risk factor for Helicobacter pylori infection: A hospital based case-control study. *Int J Diabetes Dev Ctries* 2010; **30**: 22-26 [PMID: 20431802 DOI: 10.4103/0973-3930.60008]

93   **Anastasios R**, Goritsas C, Papamihail C, Trigidou R, Garzonis P, Ferti A. Helicobacter pylori infection in diabetic patients: prevalence and endoscopic findings. *Eur J Intern Med* 2002; **13**: 376 [PMID: 12225782 DOI: 10.1016/S0953-6205(02)00094-8]

94   **He C**, Yang Z, Lu NH. Helicobacter pylori infection and diabetes: is it a myth or fact? *World J Gastroenterol* 2014; **20**: 4607-4617 [PMID: 24782613 DOI: 10.3748/wjg.v20.i16.4607]

95   **Aslan M**, Horoz M, Nazligul Y, Bolukbas C, Bolukbas FF, Selek S, Celik H, Erel O. Insulin resistance in H pylori infection and its association with oxidative stress. *World J Gastroenterol* 2006; **12**: 6865-6868 [PMID: 17106938 DOI: 10.3748/wjg.v12.i42.6865]

96   **Nasif WA**, Mukhtar MH, Nour Eldein MM, Ashgar SS. Oxidative DNA damage and oxidized low density lipoprotein in Type Ⅱ diabetes mellitus among patients with Helicobacter pylori infection. *Diabetol Metab Syndr* 2016; **8**: 34 [PMID: 27148410 DOI: 10.1186/s13098-016-0149-1]

97   **Hernández M**, Dutzan N, García-Sesnich J, Abusleme L, Dezerega A, Silva N, González FE, Vernal R, Sorsa T, Gamonal J. Host-pathogen interactions in progressive chronic periodontitis. *J Dent Res* 2011; **90**: 1164-1170 [PMID: 21471325 DOI: 10.1177/0022034511401405]

98   **Gamonal J**, Mendoza C, Espinoza I, Muñoz A, Urzúa I, Aranda W, Carvajal P, Arteaga O. Clinical attachment loss in Chilean adult population: First Chilean National Dental Examination Survey. *J Periodontol* 2010; **81**: 1403-1410 [PMID: 20629544 DOI: 10.1902/jop.2010.100148]

99   **Atanasova KR**, Yilmaz O. Looking in the Porphyromonas gingivalis cabinet of curiosities: the microbium, the host and cancer association. *Mol Oral Microbiol* 2014; **29**: 55-66 [PMID: 24506890 DOI: 10.1111/omi.12047]

100 **Genco RJ**, Van Dyke TE. Prevention: Reducing the risk of CVD in patients with periodontitis. *Nat Rev Cardiol* 2010; **7**: 479-480 [PMID: 20725103 DOI: 10.1038/nrcardio.2010.120]

101 **Han YW**, Houcken W, Loos BG, Schenkein HA, Tezal M. Periodontal disease, atherosclerosis, adverse pregnancy outcomes, and head-and-neck cancer. *Adv Dent Res* 2014; **26**: 47-55 [PMID: 24736704 DOI: 10.1177/0022034514528334]

102 **Lundberg K**, Wegner N, Yucel-Lindberg T, Venables PJ. Periodontitis in RA-the citrullinated enolase connection. *Nat Rev Rheumatol* 2010; **6**: 727-730 [PMID: 20820197 DOI: 10.1038/nrrheum.2010.139]

103 **Petersen PE**, Ogawa H. Strengthening the prevention of periodontal disease: the WHO approach. *J Periodontol* 2005; **76**: 2187-2193

[PMID: 16332229 DOI: 10.1902/jop.2005.76.12.2187]

104 **Al Sayed A**, Anand PS, Kamath KP, Patil S, Preethanath RS, Anil S. Oral Cavity as an Extragastric Reservoir of Helicobacter pylori. *ISRN Gastroenterol* 2014; **2014**: 261369 [PMID: 24701355 DOI: 10.1155/2014/261369]

105 **Zheng P**, Zhou W. Relation between periodontitis and helicobacter pylori infection. *Int J Clin Exp Med* 2015; **8**: 16741-16744 [PMID: 26629215]

106 **Sujatha S**, Jalihal UM, Sharma S. Association between periodontal disease and oral and gastric Helicobacter pylori infection. *Indian J Gastroenterol* 2015; **34**: 343-344 [PMID: 26007198 DOI: 10.1007/s12664-015-0569-0]

107 **Hu Z**, Zhang Y, Li Z, Yu Y, Kang W, Han Y, Geng X, Ge S, Sun Y. Effect of Helicobacter pylori infection on chronic periodontitis by the change of microecology and inflammation. *Oncotarget* 2016; **7**: 66700-66712 [PMID: 27602578 DOI: 10.18632/oncotarget.11449]

108 **Díaz P**, Valenzuela Valderrama M, Bravo J, Quest AFG. Helicobacter pylori and Gastric Cancer: Adaptive Cellular Mechanisms Involved in Disease Progression. *Front Microbiol* 2018; **9**: 5 [PMID: 29403459 DOI: 10.3389/fmicb.2018.00005]

109 **Valenzuela MA**, Canales J, Corvalán AH, Quest AF. Helicobacter pylori-induced inflammation and epigenetic changes during gastric carcinogenesis. *World J Gastroenterol* 2015; **21**: 12742-12756 [PMID: 26668499 DOI: 10.3748/wjg.v21.i45.12742]

110 **Taniguchi K**, Karin M. NF-κB, inflammation, immunity and cancer: coming of age. *Nat Rev Immunol* 2018; **18**: 309-324 [PMID: 29379212 DOI: 10.1038/nri.2017.142]

111 **Kanda Y**, Osaki M, Okada F. Chemopreventive Strategies for Inflammation-Related Carcinogenesis: Current Status and Future Direction. *Int J Mol Sci* 2017; **18**: pii: E867 [PMID: 28422073 DOI: 10.3390/ijms18040867]

112 **Nejati S**, Karkhah A, Darvish H, Validi M, Ebrahimpour S, Nouri HR. Influence of Helicobacter pylori virulence factors CagA and VacA on pathogenesis of gastrointestinal disorders. *Microb Pathog* 2018; **117**: 43-48 [PMID: 29432909 DOI: 10.1016/j.micpath.2018.02.016]

113 **Sitaraman R**. Allergies, Helicobacter pylori and the continental enigmas. *Front Microbiol* 2015; **6**: 578 [PMID: 26106380 DOI: 10.3389/fmicb.2015.00578]

114 **Zabaleta J**. Multifactorial etiology of gastric cancer. *Methods Mol Biol* 2012; **863**: 411-435 [PMID: 22359309 DOI: 10.1007/978-1-61779-612-8_26]

115 **Robinson K**. Helicobacter pylori-Mediated Protection against Extra-Gastric Immune and Inflammatory Disorders: The Evidence and Controversies. *Diseases* 2015; **3**: 34-55 [PMID: 28943607 DOI: 10.3390/diseases3020034]

116 **Atherton JC**, Blaser MJ. Coadaptation of Helicobacter pylori and humans: ancient history, modern implications. *J Clin Invest* 2009; **119**: 2475-2487 [PMID: 19729845 DOI: 10.1172/JCI38605]

117 **Lina TT**, Alzahrani S, Gonzalez J, Pinchuk IV, Beswick EJ, Reyes VE. Immune evasion strategies used by Helicobacter pylori. *World J Gastroenterol* 2014; **20**: 12753-12766 [PMID: 25278676 DOI: 10.3748/wjg.v20.i36.12753]

118 **Mejías-Luque R**, Gerhard M. Immune Evasion Strategies and Persistence of Helicobacter pylori. *Curr Top Microbiol Immunol* 2017; **400**: 53-71 [PMID: 28124149 DOI: 10.1007/978-3-319-50520-6_3]

119 **Morey P**, Pfannkuch L, Pang E, Boccellato F, Sigal M, Imai-Matsushima A, Dyer V, Koch M, Mollenkopf HJ, Schlaermann P, Meyer TF. Helicobacter pylori Depletes Cholesterol in Gastric Glands to Prevent Interferon Gamma Signaling and Escape the Inflammatory Response. *Gastroenterology* 2018; **154**: 1391-1404.e9 [PMID: 29273450 DOI: 10.1053/j.gastro.2017.12.008]

120 **McGee DJ**, George AE, Trainor EA, Horton KE, Hildebrandt E, Testerman TL. Cholesterol enhances Helicobacter pylori resistance to antibiotics and LL-37. *Antimicrob Agents Chemother* 2011; **55**: 2897-2904 [PMID: 21464244 DOI: 10.1128/AAC.00016-11]

121 **Du SY**, Wang HJ, Cheng HH, Chen SD, Wang LH, Wang WC. Cholesterol glucosylation by Helicobacter pylori delays internalization and arrests phagosome maturation in macrophages.

J Microbiol Immunol Infect 2016; **49**: 636-645 [PMID: 25070282 DOI: 10.1016/j.jmii.2014.05.011]

122 **Wang HJ**, Cheng WC, Cheng HH, Lai CH, Wang WC. Helicobacter pylori cholesteryl glucosides interfere with host membrane phase and affect type IV secretion system function during infection in AGS cells. *Mol Microbiol* 2012; **83**: 67-84 [PMID: 22053852 DOI: 10.1111/j.1365-2958.2011.07910.x]

123 **Wunder C**, Churin Y, Winau F, Warnecke D, Vieth M, Lindner B, Zähringer U, Mollenkopf HJ, Heinz E, Meyer TF. Cholesterol glucosylation promotes immune evasion by Helicobacter pylori. *Nat Med* 2006; **12**: 1030-1038 [PMID: 16951684 DOI: 10.1038/nm1480]

124 **Stent A**, Every AL, Chionh YT, Ng GZ, Sutton P. Superoxide dismutase from Helicobacter pylori suppresses the production of pro-inflammatory cytokines during in vivo infection. *Helicobacter* 2018; **23**: e12459 [PMID: 29235197 DOI: 10.1111/hel.12459]

125 **van Wijck Y**, de Kleijn S, John-Schuster G, Mertens TCJ, Hiemstra PS, Müller A, Smits HH, Taube C. Therapeutic Application of an Extract of Helicobacter pylori Ameliorates the Development of Allergic Airway Disease. *J Immunol* 2018; **200**: 1570-1579 [PMID: 29352004 DOI: 10.4049/jimmunol.1700987]

126 **Robinson DS**, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, Corrigan C, Durham SR, Kay AB. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992; **326**: 298-304 [PMID: 1530827 DOI: 10.1056/NEJM199201303260504]

127 **Zhang M**, Berndt BE, Eaton KA, Rathinavelu S, Pierzchala A, Kao JY. Helicobacter pylori-pulsed dendritic cells induce H. pylori-specific immunity in mice. *Helicobacter* 2008; **13**: 200-208 [PMID: 18466395 DOI: 10.1111/j.1523-5378.2008.00606.x]

128 **Kao JY**, Zhang M, Miller MJ, Mills JC, Wang B, Liu M, Eaton KA, Zou W, Berndt BE, Cole TS, Takeuchi T, Owyang SY, Luther J. Helicobacter pylori immune escape is mediated by dendritic cell-induced Treg skewing and Th17 suppression in mice. *Gastroenterology* 2010; **138**: 1046-1054 [PMID: 19931266 DOI: 10.1053/j.gastro.2009.11.043]

129 **Mitchell P**, Germain C, Fiori PL, Khamri W, Foster GR, Ghosh S, Lechler RI, Bamford KB, Lombardi G. Chronic exposure to Helicobacter pylori impairs dendritic cell function and inhibits Th1 development. *Infect Immun* 2007; **75**: 810-819 [PMID: 17101659 DOI: 10.1128/IAI.00228-06]

130 **Hussain K**, Letley DP, Greenaway AB, Kenefeck R, Winter JA, Tomlinson W, Rhead J, Staples E, Kaneko K, Atherton JC, Robinson K. Helicobacter pylori-Mediated Protection from Allergy Is Associated with IL-10-Secreting Peripheral Blood Regulatory T Cells. *Front Immunol* 2016; **7**: 71 [PMID: 27014260 DOI: 10.3389/fimmu.2016.00071]

131 **Arnold IC**, Dehzad N, Reuter S, Martin H, Becher B, Taube C, Müller A. Helicobacter pylori infection prevents allergic asthma in mouse models through the induction of regulatory T cells. *J Clin Invest* 2011; **121**: 3088-3093 [PMID: 21737881 DOI: 10.1172/JCI45041]

132 **Arnold IC**, Lee JY, Amieva MR, Roers A, Flavell RA, Sparwasser T, Müller A. Tolerance rather than immunity protects from Helicobacter pylori-induced gastric preneoplasia. *Gastroenterology* 2011; **140**: 199-209 [PMID: 20600031 DOI: 10.1053/j.gastro.2010.06.047]

133 **Bagheri N**, Shirzad H, Elahi S, Azadegan-Dehkordi F, Rahimian G, Shafigh M, Rashidii R, Sarafnejad A, Rafieian-Kopaei M, Faridani R, Tahmasbi K, Kheiri S, Razavi A. Downregulated regulatory T cell function is associated with increased peptic ulcer in Helicobacter pylori-infection. *Microb Pathog* 2017; **110**: 165-175 [PMID: 28666843 DOI: 10.1016/j.micpath.2017.06.040]

134 **Zhou S**, Huang Y, Liang B, Dong H, Yao S, Chen Y, Xie Y, Long Y, Gong S, Zhou Z. Systemic and mucosal pre-administration of recombinant Helicobacter pylori neutrophil-activating protein prevents ovalbumin-induced allergic asthma in mice. *FEMS Microbiol Lett* 2017; **364**: pii: fnw288 [PMID: 28087613 DOI: 10.1093/femsle/fnw288]

135 **Tran LS**, Chonwerawong M, Ferrero RL. Regulation and functions of inflammasome-mediated cytokines in Helicobacter pylori

136 **Gong Y**, Tao L, Jing L, Liu D, Hu S, Liu W, Zhou N, Xie Y. Association of TLR4 and Treg in Helicobacter pylori Colonization and Inflammation in Mice. *PLoS One* 2016; **11**: e0149629 [PMID: 26901645 DOI: 10.1371/journal.pone.0149629]

137 **Yuan X**, Zhou Y, Wang W, Li J, Xie G, Zhao Y, Xu D, Shen L. Activation of TLR4 signaling promotes gastric cancer progression by inducing mitochondrial ROS production. *Cell Death Dis* 2013; **4**: e794 [PMID: 24030146 DOI: 10.1038/cddis.2013.334]

138 **Kawahara T**, Teshima S, Oka A, Sugiyama T, Kishi K, Rokutan K. Type I Helicobacter pylori lipopolysaccharide stimulates toll-like receptor 4 and activates mitogen oxidase 1 in gastric pit cells. *Infect Immun* 2001; **69**: 4382-4389 [PMID: 11401977 DOI: 10.1128/IAI.69.7.4382-4389.2001]

139 **Lepper PM**, Triantafilou M, Schumann C, Schneider EM, Triantafilou K. Lipopolysaccharides from Helicobacter pylori can act as antagonists for Toll-like receptor 4. *Cell Microbiol* 2005; **7**: 519-528 [PMID: 15760452 DOI: 10.1111/j.1462-5822.2005.00482.x]

140 **Kawahara T**, Kuwano Y, Teshima-Kondo S, Sugiyama T, Kawai T, Nikawa T, Kishi K, Rokutan K. Helicobacter pylori lipopolysaccharide from type I, but not type II strains, stimulates apoptosis of cultured gastric mucosal cells. *J Med Invest* 2001; **48**: 167-174 [PMID: 11694956]

141 **Sun X**, Zhang M, El-Zataari M, Owyang SY, Eaton KA, Liu M, Chang YM, Zou W, Kao JY. TLR2 mediates Helicobacter pylori-induced tolerogenic immune response in mice. *PLoS One* 2013; **8**: e74595 [PMID: 24058595 DOI: 10.1371/journal.pone.0074595]

142 **Otani K**, Tanigawa T, Watanabe T, Nadatani Y, Sogawa M, Yamagami H, Shiba M, Watanabe K, Tominaga K, Fujiwara Y, Arakawa T. Toll-like receptor 9 signaling has anti-inflammatory effects on the early phase of Helicobacter pylori-induced gastritis. *Biochem Biophys Res Commun* 2012; **426**: 342-349 [PMID: 22940550 DOI: 10.1016/j.bbrc.2012.08.080]

143 **Teymournejad O**, Mobarez AM, Hassan ZM, Moazzeni SM, Ahmadabad HN. In vitro suppression of dendritic cells by Helicobacter pylori OipA. *Helicobacter* 2014; **19**: 136-143 [PMID: 24495278 DOI: 10.1111/hel.12107]

144 **Hock BD**, McKenzie JL, Keenan JI. Helicobacter pylori outer membrane vesicles inhibit human T cell responses via induction of monocyte COX-2 expression. *Pathog Dis* 2017; **75**: ftx034 [PMID: 28430970 DOI: 10.1093/femspd/ftx034]

145 **Winter J**, Letley D, Rhead J, Atherton J, Robinson K. Helicobacter pylori membrane vesicles stimulate innate pro- and anti-inflammatory responses and induce apoptosis in Jurkat T cells. *Infect Immun* 2014; **82**: 1372-1381 [PMID: 24421041 DOI: 10.1128/IAI.01443-13]

146 **Owyang SY**, Luther J, Owyang CC, Zhang M, Kao JY. Helicobacter pylori DNA's anti-inflammatory effect on experimental colitis. *Gut Microbes* 2012; **3**: 168-171 [PMID: 22356863 DOI: 10.4161/gmic.19181]

147 **Engstrand L**, Lindberg M. Helicobacter pylori and the gastric microbiota. *Best Pract Res Clin Gastroenterol* 2013; **27**: 39-45 [PMID: 23768551 DOI: 10.1016/j.bpg.2013.03.016]

148 **Kato S**, Fujimura S, Kimura K, Nishio T, Hamada S, Minoura T, Oda M. Non-Helicobacter bacterial flora rarely develops in the gastric mucosal layer of children. *Dig Dis Sci* 2006; **51**: 641-646 [PMID: 16614982 DOI: 10.1007/s10620-006-3185-0]

149 **Sanduleanu S**, Jonkers D, De Bruine A, Hameeteman W, Stockbrügger RW. Non-Helicobacter pylori bacterial flora during acid-suppressive therapy: differential findings in gastric juice and gastric mucosa. *Aliment Pharmacol Ther* 2001; **15**: 379-388 [PMID: 11207513 DOI: 10.1046/j.1365-2036.2001.00888.x]

150 **Sharma BK**, Santana IA, Wood EC, Walt RP, Pereira M, Noone P, Smith PL, Walters CL, Pounder RE. Intragastric bacterial activity and nitrosation before, during, and after treatment with omeprazole. *Br Med J* (Clin Res Ed) 1984; **289**: 717-719 [PMID: 6434053 DOI: 10.1136/bmj.289.6447.717]

151 **Zilberstein B**, Quintanilha AG, Santos MA, Pajecki D, Moura EG, Alves PR, Maluf Filho F, de Souza JA, Gama-Rodrigues J. Digestive tract microbiota in healthy volunteers. *Clinics*

(Sao Paulo) 2007; **62**: 47-54 [PMID: 17334549 DOI: 10.1590/S1807-59322007000100008]

152 **Bik EM**, Eckburg PB, Gill SR, Nelson KE, Purdom EA, Francois F, Perez-Perez G, Blaser MJ, Relman DA. Molecular analysis of the bacterial microbiota in the human stomach. *Proc Natl Acad Sci USA* 2006; **103**: 732-737 [PMID: 16407106 DOI: 10.1073/pnas.0506655103]

153 **Delgado S**, Cabrera-Rubio R, Mira A, Suárez A, Mayo B. Microbiological survey of the human gastric ecosystem using culturing and pyrosequencing methods. *Microb Ecol* 2013; **65**: 763-772 [PMID: 23397369 DOI: 10.1007/s00248-013-0192-5]

154 **Dicksved J**, Lindberg M, Rosenquist M, Enroth H, Jansson JK, Engstrand L. Molecular characterization of the stomach microbiota in patients with gastric cancer and in controls. *J Med Microbiol* 2009; **58**: 509-516 [PMID: 19273648 DOI: 10.1099/jmm.0.007302-0]

155 **Li XX**, Wong GL, To KF, Wong VW, Lai LH, Chow DK, Lau JY, Sung JJ, Ding C. Bacterial microbiota profiling in gastritis without Helicobacter pylori infection or non-steroidal anti-inflammatory drug use. *PLoS One* 2009; **4**: e7985 [PMID: 19956741 DOI: 10.1371/journal.pone.0007985]

156 **Maldonado-Contreras A**, Goldfarb KC, Godoy-Vitorino F, Karaoz U, Contreras M, Blaser MJ, Brodie EL, Dominguez-Bello MG. Structure of the human gastric bacterial community in relation to Helicobacter pylori status. *ISME J* 2011; **5**: 574-579 [PMID: 20927139 DOI: 10.1038/ismej.2010.149]

157 **Tan MP**, Kaparakis M, Galic M, Pedersen J, Pearse M, Wijburg OL, Janssen PH, Strugnell RA. Chronic Helicobacter pylori infection does not significantly alter the microbiota of the murine stomach. *Appl Environ Microbiol* 2007; **73**: 1010-1013 [PMID: 17142378 DOI: 10.1128/AEM.01675-06]

158 **Schulz C**, Schütte K, Koch N, Vilchez-Vargas R, Wos-Oxley ML, Oxley APA, Vital M, Malfertheiner P, Pieper DH. The active bacterial assemblages of the upper GI tract in individuals with and without Helicobacter infection. *Gut* 2018; **67**: 216-225 [PMID: 27920199 DOI: 10.1136/gutjnl-2016-312904]

159 **Dong Q**, Xin Y, Wang L, Meng X, Yu X, Lu L, Xuan S. Characterization of Gastric Microbiota in Twins. *Curr Microbiol* 2017; **74**: 224-229 [PMID: 27957630 DOI: 10.1007/s00284-016-1176-8]

160 **Martin ME**, Bhatnagar S, George MD, Paster BJ, Canfield DR, Eisen JA, Solnick JV. The impact of Helicobacter pylori infection on the gastric microbiota of the rhesus macaque. *PLoS One* 2013; **8**: e76375 [PMID: 24116104 DOI: 10.1371/journal.pone.0076375]

161 **Brawner KM**, Kumar R, Serrano CA, Ptacek T, Lefkowitz E, Morrow CD, Zhi D, Kyanam-Kabir-Baig KR, Smythies LE, Harris PR, Smith PD. Helicobacter pylori infection is associated with an altered gastric microbiota in children. *Mucosal Immunol* 2017; **10**: 1169-1177 [PMID: 28120843 DOI: 10.1038/mi.2016.131]

162 **Bauerfeind P**, Garner R, Dunn BE, Mobley HL. Synthesis and activity of Helicobacter pylori urease and catalase at low pH. *Gut* 1997; **40**: 25-30 [PMID: 9155571 DOI: 10.1136/gut.40.1.25]

163 **Wen Y**, Feng J, Scott DR, Marcus EA, Sachs G. The HP0165-HP0166 two-component system (ArsRS) regulates acid-induced expression of HP1186 alpha-carbonic anhydrase in Helicobacter pylori by activating the pH-dependent promoter. *J Bacteriol* 2007; **189**: 2426-2434 [PMID: 17220228 DOI: 10.1128/JB.01492-06]

164 **Williams CL**, Preston T, Hossack M, Slater C, McColl KE. Helicobacter pylori utilises urea for amino acid synthesis. *FEMS Immunol Med Microbiol* 1996; **13**: 87-94 [PMID: 8821403 DOI: 10.1111/j.1574-695X.1996.tb00220.x]

165 **Ziebarth D**, Spiegelhalder B, Bartsch H. N-nitrosation of medicinal drugs catalysed by bacteria from human saliva and gastro-intestinal tract, including Helicobacter pylori. *Carcinogenesis* 1997; **18**: 383-389 [PMID: 9054633 DOI: 10.1093/carcin/18.2.383]

166 **Aebischer T**, Fischer A, Walduck A, Schlötelburg C, Lindig M, Schreiber S, Meyer TF, Bereswill S, Göbel UB. Vaccination prevents Helicobacter pylori-induced alterations of the gastric flora in mice. *FEMS Immunol Med Microbiol* 2006; **46**: 221-229 [PMID: 16487303 DOI: 10.1111/j.1574-695X.2005.00024.x]

167 **Parsons BN**, Ijaz UZ, D'Amore R, Burkitt MD, Eccles R, Lenzi L, Duckworth CA, Moore AR, Tiszlavicz L, Varro A, Hall N, Pritchard DM. Comparison of the human gastric microbiota in hypochlorhydric states arising as a result of Helicobacter pylori-induced atrophic gastritis, autoimmune atrophic gastritis and proton pump inhibitor use. *PLoS Pathog* 2017; **13**: e1006653 [PMID: 29095917 DOI: 10.1371/journal.ppat.1006653]

168 **Hansson LE**, Nyrén O, Hsing AW, Bergström R, Josefsson S, Chow WH, Fraumeni JF Jr, Adami HO. The risk of stomach cancer in patients with gastric or duodenal ulcer disease. *N Engl J Med* 1996; **335**: 242-249 [PMID: 8657240 DOI: 10.1056/NEJM199607253350404]

169 **Uemura N**, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ. Helicobacter pylori infection and the development of gastric cancer. *N Engl J Med* 2001; **345**: 784-789 [PMID: 11556297 DOI: 10.1056/NEJMoa001999]

170 **Guerre J**, Vedel G, Gaudric M, Paul G, Cornuau J. Bacterial flora in gastric juice taken at endoscopy in 93 normal subjects. *Pathol Biol* (Paris) 1986; **34**: 57-60 [PMID: 3517771]

171 **Espinoza JL**, Matsumoto A, Tanaka H, Matsumura I. Gastric microbiota: An emerging player in Helicobacter pylori-induced gastric malignancies. *Cancer Lett* 2018; **414**: 147-152 [PMID: 29138097 DOI: 10.1016/j.canlet.2017.11.009]

172 **Goddard AF**, Spiller RC. The effect of omeprazole on gastric juice viscosity, pH and bacterial counts. *Aliment Pharmacol Ther* 1996; **10**: 105-109 [PMID: 8871450 DOI: 10.1111/j.1365-2036.1996.tb00183.x]

173 **Navabi N**, Johansson ME, Raghavan S, Lindén SK. Helicobacter pylori infection impairs the mucin production rate and turnover in the murine gastric mucosa. *Infect Immun* 2013; **81**: 829-837 [PMID: 23275091 DOI: 10.1128/IAI.01000-12]

174 **Manson JM**, Rauch M, Gilmore MS. The commensal microbiology of the gastrointestinal tract. *Adv Exp Med Biol* 2008; **635**: 15-28 [PMID: 18841700 DOI: 10.1007/978-0-387-09550-9_2]

175 **Akcam M**, Ozdem S, Yilmaz A, Gultekin M, Artan R. Serum ferritin, vitamin B(12), folate, and zinc levels in children infected with Helicobacter pylori. *Dig Dis Sci* 2007; **52**: 405-410 [PMID: 17211708 DOI: 10.1007/s10620-006-9422-8]

176 **Capurso G**, Marignani M, Delle Fave G, Annibale B. Iron-deficiency anemia in premenopausal women: why not consider atrophic body gastritis and Helicobacter pylori role? *Am J Gastroenterol* 1999; **94**: 3084-3085 [PMID: 10520890 DOI: 10.1111/j.1572-0241.1999.03084.x]

177 **Gao XX**, Ge HM, Zheng WF, Tan RX. NMR-based metabonomics for detection of Helicobacter pylori infection in gerbils: which is more descriptive. *Helicobacter* 2008; **13**: 103-111 [PMID: 18321300 DOI: 10.1111/j.1523-5378.2008.00590.x]

178 **Klymiuk I**, Bilgilier C, Stadlmann A, Thannesberger J, Kastner MT, Högenauer C, Püspök A, Biowski-Frotz S, Schrutka-Kölbl C, Thallinger GG, Steininger C. The Human Gastric Microbiome Is Predicated upon Infection with Helicobacter pylori. *Front Microbiol* 2017; **8**: 2508 [PMID: 29312210 DOI: 10.3389/fmicb.2017.02508]

179 **Jones TA**, Hernandez DZ, Wong ZC, Wandler AM, Guillemin K. The bacterial virulence factor CagA induces microbial dysbiosis that contributes to excessive epithelial cell proliferation in the Drosophila gut. *PLoS Pathog* 2017; **13**: e1006631 [PMID: 29049360 DOI: 10.1371/journal.ppat.1006631]

180 **Yap TW**, Gan HM, Lee YP, Leow AH, Azmi AN, Francois F, Perez-Perez GI, Loke MF, Goh KL, Vadivelu J. Helicobacter pylori Eradication Causes Perturbation of the Human Gut Microbiome in Young Adults. *PLoS One* 2016; **11**: e0151893 [PMID: 26991500 DOI: 10.1371/journal.pone.0151893]

181 **Heimesaat MM**, Fischer A, Plickert R, Wiedemann T, Loddenkemper C, Göbel UB, Bereswill S, Rieder G. Helicobacter pylori induced gastric immunopathology is associated with distinct microbiota changes in the large intestines of long-term infected Mongolian gerbils. *PLoS One* 2014; **9**: e100362 [PMID: 24941045 DOI: 10.1371/journal.pone.0100362]

182 **Majlessi L**, Sayes F, Bureau JF, Pawlik A, Michel V, Jouvion G, Huerre M, Severgnini M, Consolandi C, Peano C, Brosch R, Touati

E, Leclerc C. Colonization with Helicobacter is concomitant with modified gut microbiota and drastic failure of the immune control of Mycobacterium tuberculosis. *Mucosal Immunol* 2017; **10**: 1178-1189 [PMID: 28145441 DOI: 10.1038/mi.2016.140]

183 **Gebert B**, Fischer W, Weiss E, Hoffmann R, Haas R. Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation. *Science* 2003; **301**: 1099-1102 [PMID: 12934009 DOI: 10.1126/science.1086871]

184 **Wüstner S**, Mejías-Luque R, Koch MF, Rath E, Vieth M, Sieber SA, Haller D, Gerhard M. Helicobacter pylori γ-glutamyltranspeptidase impairs T-lymphocyte function by compromising metabolic adaption through inhibition of cMyc and IRF4 expression. *Cell Microbiol* 2015; **17**: 51-61 [PMID: 25087912 DOI: 10.1111/cmi.12335]

185 **Khosravi Y**, Loke MF, Goh KL, Vadivelu J. Proteomics Analysis Revealed that Crosstalk between Helicobacter pylori and Streptococcus mitis May Enhance Bacterial Survival and Reduces Carcinogenesis. *Front Microbiol* 2016; **7**: 1462 [PMID: 27695448 DOI: 10.3389/fmicb.2016.01462]

186 **Boyanova L**, Gergova G, Markovska R, Yordanov D, Mitov I. Bacteriocin-like inhibitory activities of seven Lactobacillus delbrueckii subsp. bulgaricus strains against antibiotic susceptible and resistant Helicobacter pylori strains. *Lett Appl Microbiol* 2017; **65**: 469-474 [PMID: 28975642 DOI: 10.1111/lam.12807]

187 **Salas-Jara MJ**, Sanhueza EA, Retamal-Díaz A, González C,

Urrutia H, García A. Probiotic Lactobacillus fermentum UCO-979C biofilm formation on AGS and Caco-2 cells and Helicobacter pylori inhibition. *Biofouling* 2016; **32**: 1245-1257 [PMID: 27834106 DOI: 10.1080/08927014.2016.1249367]

188 **Zheng PX**, Fang HY, Yang HB, Tien NY, Wang MC, Wu JJ. Lactobacillus pentosus strain LPS16 produces lactic acid, inhibiting multidrug-resistant Helicobacter pylori. *J Microbiol Immunol Infect* 2016; **49**: 168-174 [PMID: 24874430 DOI: 10.1016/j.jmii.2014.04.014]

189 **Elitsur Y**, Neace C, Werthammer MC, Triest WE. Prevalence of CagA, VacA antibodies in symptomatic and asymptomatic children with Helicobacter pylori infection. *Helicobacter* 1999; **4**: 100-105 [PMID: 10382123 DOI: 10.1046/j.1523-5378.1999.98530.x]

190 **Hooper LV**, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science* 2012; **336**: 1268-1273 [PMID: 22674334 DOI: 10.1126/science.1223490]

191 **Rolig AS**, Cech C, Ahler E, Carter JE, Ottemann KM. The degree of Helicobacter pylori-triggered inflammation is manipulated by preinfection host microbiota. *Infect Immun* 2013; **81**: 1382-1389 [PMID: 23429529 DOI: 10.1128/IAI.00044-13]

192 **Pei X**, Ouyang X, He L, Cao C, Luan Q, Suda R. A 4-year prospective study of the progression of periodontal disease in a rural Chinese population. *J Dent* 2015; **43**: 192-200 [PMID: 25527246 DOI: 10.1016/j.jdent.2014.12.008]

REVIEW

Proton therapy for hepatocellular carcinoma: Current knowledges and future perspectives

Gyu Sang Yoo, Jeong Il Yu, Hee Chul Park

Gyu Sang Yoo, Jeong Il Yu, Hee Chul Park, Department of Radiation Oncology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 06351, South Korea

Hee Chul Park, Department of Medical Device Management and Research, Samsung Advanced Institute for Health Sciences and Technology, Sungkyunkwan University, Seoul 06351, South Korea

ORCID number: Gyu Sang Yoo (0000-0002-5542-5263); Jeong Il Yu (0000-0002-2009-7263); Hee Chul Park (0000-0003-0385-9681).

Abstract

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death, as few patients can be treated with currently available curative local modalities. In patients with HCC where curative modalities are not feasible, radiation therapy (RT) has emerged as an alternative or combination therapy. With the development of various technologies, RT has been increasingly used for the management of HCC. Among these advances, proton beam therapy (PBT) has several unique physical properties that give it a finite range in a distal direction, and thus no exit dose along the beam path. Therefore, PBT has dosimetric advantages compared with X-ray therapy for the treatment of HCC. Indeed, various reports in the literature have described the favorable clinical outcomes and improved safety of PBT for HCC patients compared with X-ray therapy. However, there are some technical issues regarding the use of PBT in HCC, including uncertainty of organ motion and inaccuracy during calculation of tissue density and beam range, all of which may reduce the robustness of a PBT treatment plan. In this review, we discuss the physical properties, current clinical data, technical issues, and future perspectives on PBT for the treatment of HCC.

Key words: Radiation therapy; Hepatocellular carcinoma; Proton beam therapy; X-ray therapy

Core tip: Radiation therapy (RT) is being increasingly

utilized for the treatment of hepatocellular carcinoma (HCC). As RT technology develops, proton beam therapy (PBT) has emerged as a method that affords dosimetric advantages compared with X-ray therapy due to its physical properties, including lack of an exit dose along the beam path. Clinical experience with PBT for HCC is accumulating rapidly, and the effectiveness and safety of PBT has been validated. In this review, we discuss the physical properties, current clinical data, technical issues, and future perspectives on PBT for the treatment of HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver, accounting for 90% of all liver cancers[1]. HCC is the fifth most common cancer worldwide and the second leading cause of cancer-related death[2]. Although there is a relatively high incidence of HCC in South-East Asia, rates have also been increasing in North America and Western Europe[3]. HCC has various etiological factors including hepatitis B and C infection, alcohol consumption, liver cirrhosis, non-alcoholic fatty liver disease, obesity, diabetes, aflatoxin exposure, and hereditary disorders such as hemochromatosis and alpha-1-antitrypsin deficiency[4-6].

Although there have been improvements in the management of HCC[7,8], application of curative local modalities such as surgical resection, transplantation, and radiofrequency ablation are often limited due to tumor extent, tumor location, or other patient-related factors[9-11]. Therefore, the prognosis of HCC continues to be unsatisfactory, with a five-year survival of less than 20%, despite improvement in overall survival (OS) by non-curative modalities including chemoembolization and sorafenib[12]. For this reason, radiation therapy (RT) as a local modality has emerged as an alternative/combination treatment approach[13-15]. As the prognosis of HCC depends on sustained control of tumor size[16], there has been growing interest in use of external beam RT[13,14,17,18]. More specifically, advances in proton beam therapy (PBT) have brought additional innovation to the field of RT for the treatment of HCC[12,19,20].

PBT has a dosimetric advantage compared to X-ray therapy due to the physical properties of the technique[21,22]. Therefore, PBT is potentially more beneficial in sparing organs-at risk (OARs), especially for cases where the tumor is proximal to OARs. For liver tumors, the tolerance of surrounding normal liver, biliary tracts, and gastrointestinal (GI) structures is the main limiting factor for dose escalation[23,24]. For these reasons, there has been increasing interest in the use of PBT for treating liver

tumors, and clinical experiences and evidences regarding the advantages of PBT continue to accumulate[12,20,25,26]. Here, we review the current knowledge and future perspectives on PBT for HCC.

PHYSICAL CHARACTERISTICS AND DOSIMETRIC ADVANTAGES OF PBT

The deposition of X-ray dose decreases gradually along the beam path with increasing beam depth[27]. As a result, an exit dose is inevitably deposited in adjacent normal tissues. Intensity-modulated RT (IMRT) or volumetric-modulated arc therapy (VMAT) techniques can be used to obtain more conformal dose distributions; however, these methods are still unable to avoid low-dose deposition at the distal area of the beam path. Because of these unfavorable characteristics, RT has had a very limited role in the treatment of HCC in patients whose livers are mostly cirrhotic or poorly functioning, as these patients are most vulnerable to radiation-induced liver disease (RILD)[28,29].

Compared to X-ray beams, a proton beam has a finite range of energy deposition and loses most of its energy within a very short distance at the end of the beam range (Figure 1). These results in a sharp rise and fall in energy absorption, known as the Bragg peak. Bragg peaks can be superposed to provide wider depth coverage, so-called "spread-out Bragg peak beams (SOBP)"[27]. Furthermore, due to the absence of an exit dose, PBT has dosimetric advantages compared with 3-dimentional conformal RT or even IMRT or VMAT[21,22]. Therefore, PBT has the potential to both decrease the risk of RILD and allow for safer escalation of radiation dose[30].

BIOLOGICAL CHARACTERISTICS OF PBT

The proton beams cause deoxyribose-nucleotide acid (DNA) damage and cytotoxicity by direct collisions with DNA molecules and by the generation of radical oxygen specimen (ROS) causing indirect DNA damage[31]. The efficiency of DNA damage is better for PBT than X-ray therapy due to relatively high energies of PBT used for RT[32]. Therefore, the PBT is more effect for a given prescription dose than X-rays[33]. The concept of the relative biological effectiveness (RBE) is useful to consider this issue. The definition of RBE is the ratio of the proton dose to the photon dose for a given level of effect, and proton has higher value of RBE than X-ray. The RBE of the proton beams depends on various parameters such as dose, considered endpoint, linear energy transfer, α/β of the tumor or tissue, and the positions of the SOBP[34]. There is trends that the RBE increases as decreasing α/β, decreasing dose, increasing LET, and approaching the distal edge and distal fall-off of the SOBP[35-37]. Because these dependencies are considered negligible compared with the uncertainties for tissue density[33], beam ranges, and patients, up to date, fixed value of 1.1 which is
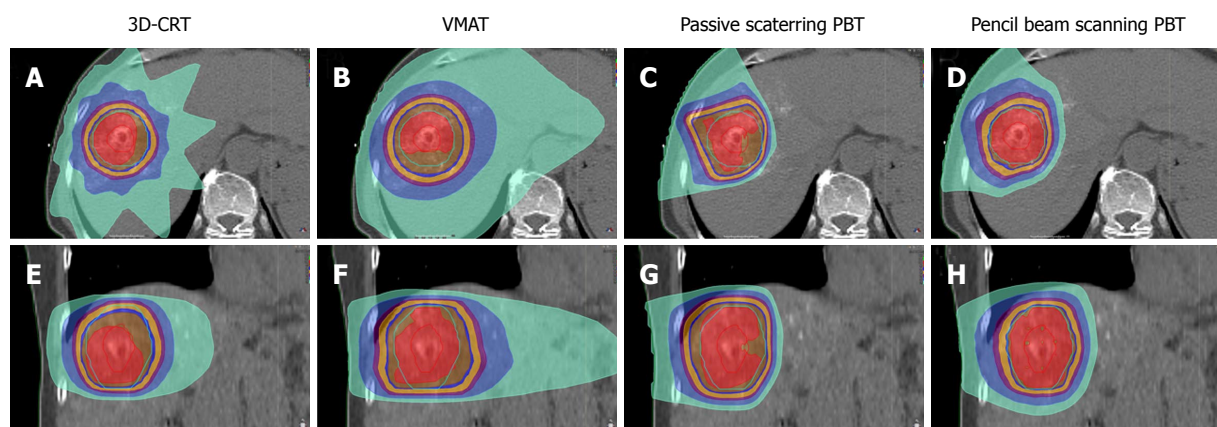
| 3D-CRT | VMAT | Passive scaterring PBT | Pencil beam scanning PBT |

Figure 1 Radiation dose distribution according to technique of radiation therapy. Axial views of A: 3-dimentional conformal radiation therapy (3D-CRT); B: Volumetric arc therapy (VMAT); C: Passive scattering proton beam therapy (PBT); D: Pencil beam scanning PBT; sagittal views of E: 3D-CRT; F: VMAT; G: PBT with wobbling technique; H: PBT with pencil beam scanning technique. There are low dose distributions in 3D-CRT (A, E) and VMAT (B, F) due to the exit dose.

based on various *in vivo* and *in vitro* data has been commonly used as universal value of RBE for proton beam[38]. However, the clinical impact of variability in RBE has been being investigated and with precise modeling variable RBE can be used for further optimization of PBT plan[33].

CLINICAL OUTCOMES OF PBT FOR HCC

The majority of clinical outcomes regarding PBT for HCC have been reported from Eastern Asia and the United States (US) due to the high incidence of HCC and long-term experience in the clinical use of PBT [19,20]. An overview of the published studies of PBT for HCC is summarized in Table 1. The Proton Medical Research Center (PMRC) of the University of Tsukuba, Japan, developed three PBT protocols according to tumor location with respect to the porta hepatis and GI OARs[30,39-42]. Specifically, for peripheral tumors > 2 cm from both GI tract and the porta hepatis, a 66-Gy relative biologic equivalent (GyE) in 10 fractions is delivered. Likewise, tumors within 2 cm of the GI tract can be treated with 77.0 GyE in 35 fractions, while those within 2 cm of the porta hepatis are treated with 72.6 GyE in 22 fractions. In those studies, the authors reported local control (LC) in the range of 88% to 95%, which was similar among various dose and fractionations. In addition, the authors reported 3-year OS rates ranging from 45% to 65%[30,39-42]. They also found that liver function was a significant prognostic factor associated with OS rates, and that OS was better in patients with a Child-Pugh (CP) A score compared with CP B.

In the US, researchers at Loma Linda University performed a phase Ⅱ trial of PBT in 76 patients with HCC and liver cirrhosis[43]. Patients with extrahepatic metastases, tense ascites, or more than three tumors were excluded. The progression-free survival (PFS) at 3 years for patients meeting the Milan criteria was 60%. Liver transplantation was performed in 18 patients whose eventual 3-year OS rate was 70%; however, the OS rate of patients who did not undergo liver transplantation

was 10%. In these patients, multivariate analysis identified the Milan criteria as the only independent factor associated with OS.

Researchers from Loma Linda University also recently reported a preliminary analysis of a randomized trial of TACE versus PBT[44]. In that study, a total of 69 patients who were diagnosed of HCC and met the Milan or San Francisco transplant criteria were enrolled and randomized to either TACE (*n* = 36) or PBT (*n* = 33). The dose fractionation regimen of PBT was 70.2 GyE in 15 fractions. An interim analysis showed a trend toward improved 2-year LC (88% *vs* 45%) and PFS (48% *vs* 31%) favoring PBT, although the difference between the two methods was not statistically significant. Another phase Ⅱ trial of PBT for HCC (*n* = 44) and intrahepatic cholangiocarcinoma (*n* = 37) performed as a multi-center trial in the US[25] reported the use of dose fractionation regimens of intention of 67.6 GyE in 15 fractions for tumors > 2 cm from the porta hepatis and 58.05 GyE in 15 fractions for those within 2 cm of porta hepatis. In that study, the median dose actually delivered was 58.05 GyE (range, 15.1 to 67.5 GyE) in 15 fractions. The LC and OS rates at 2 years were 95% and 63%, respectively, supporting ongoing phase Ⅲ trials of RBT in HCC.

In South Korea, the National Cancer Center performed a phase Ⅰ dose-escalation study for PBT in HCC comprising 60 GyE in 20 fractions, 66 GyE in 22 fractions, or 72 GyE in 24 fractions[45]. The complete response (CR) rates of PBT were 62.5%, 57.1%, and 100% according to increasing dose level, respectively. In addition, the 3-year LC rate was 79.9%, which was significantly higher in patients with CR than in those with non-CR (90% *vs* 40%, *P* = 0.003). None of the patients experienced liver toxicities greater than grade 2.

PBT FOR HCC IN SPECIAL SITUATIONS

Although various clinical studies have demonstrated the effectiveness and safety of PBT in the treatment for HCC,

Table 1 Studies of proton beam therapy for hepatocellular carcinoma

Study	Patients (*n*)	CTP score	Tumor size (cm)	PVTT	RT regimen (GyE/fractions)	LC	PFS	OS
Tsukuba (2008)[39]	53	A: 87% B: 11% C: 2%	≤ 3: 25% > 3 - < 5: 34% ≥ 5 - < 10: 34% ≥ 10: 8%	28%	72.6/22 (80.5 Gy EQD2)	94% at 2 yr 86% at 3 yr	38% at 2 yr 25% at 3 yr	2-yr 57% 3-yr 45%
Tsukuba (2009)[40]	51	A: 80% B: 20%	Median 2.8 (range, 0.8 to 9.3) ≤ 5: 88% > 5: 12%	NA	66.0/10 (91.3 Gy EQD2)	95% at 3 yr 88% at 5 yr	NA	3-yr 49% 5-yr 39%
Tsukuba (2009)[41]	318	A: 74% B: 24% C: 2%	NA	14%	66.0/10 (91.3 Gy EQD2): 32.7% 72.6/22 (80.5 Gy EQD2): 26.7% 77.0/35 (78.3 Gy EQD2): 20.8%	NA	NA	3-yr 65% 5-yr 45%
Tsukuba (2011)[42]	47	A: 75% B: 19% C: 6%	NA	15%	72.6/22 (80.5 Gy EQD2): 34.0% 77.0/35 (78.3 Gy EQD2): 27.7%	88% at 3 yr 88% at 4 yr	IHRFS 1-yr 66% 3-yr 40% 4-yr 17%	3-yr 50% 4-yr 34%
Loma Linda (2011)[43]	76	A: 29% B: 47% C: 24%	Mean 5.5 ≤ 2: 7% > 2 - < 5: 45% ≥ 5 - < 10: 43% ≥ 10: 5%	5%	63.0/15 (74.6 Gy EQD2)	70% at 3 yr	80%	Median 36 mo
NCC (2015)[45]	27	A: 89% B: 11%	≤ 5: 81% > 5: 19%	NA	60.0/20 (65.0 Gy EQD2): 29.6% 66.0/22 (71.5 Gy EQD2): 25.9% 72.0/24 (78.0 Gy EQD2): 44.4%	80% at 3 yr 64% at 5 yr	3-yr 17% 5-yr 0	3-yr 56% 5-yr 42%
Multiple United States institutions (2016)[25]	44	A: 73% B: 20% NA: 7%	Median 5.0 (range, 1.9 to 12.0)	30%	Median 58.05/15 (67.1 Gy EQD2) (range, 15.1-67.5/15)	95% at 2 yr	1-yr 56% 2-yr 40%	1-yr 77% 2-yr 63%

CTP: Child-Turcotte-Pugh; PVTT: Portal vein tumor thrombosis; RT: Radiation therapy; GyE: Gy equivalent; LC: Local control; PFS: Progression free survival; OS: Overall survival; EQD2: Equivalent dose in 2 Gy; NA: Not applicable; NCC: National Cancer Center in South Korea.

the indications for PBT have not yet been determined and continue to evolve. Because of dosimetric advantages, PBT has the potential to be applicable to more complicated cases ineligible for X-ray therapy, such as those with a history of hepatic RT, vascular invasion of the tumor, extremely large tumor burden, and/or poor liver function.

RE-IRRADIATION WITH PBT FOR HCC

RT has played a role as a salvage treatment for recurrent HCC refractory or ineligible for other modalities[46-48]. RT can also be applied to recurrent HCC previously irradiated when there is no appropriate local salvage modality. However, despite the development of modern RT techniques, re-irradiation in HCC remains a challenging issue due to the risk of RILD and GI toxicity[49-51]. Therefore, very few studies have evaluated the feasibility and effectiveness of hepatic re-irradiation for HCC[51]. Nevertheless, PBT has been investigated as a salvage modality for previously irradiated HCC. Scientists at PMRC reported on the outcomes of repeated PBT for HCC[52]. In that study, 27 patients with 68 lesions received 2 or more courses of PBT, and the median interval between the first and second courses was 24.5 mo (range, 3.3 to 79.8 mo). Likewise, the median dose was 72 Gy in 16 fractions and 66 Gy in 16 fractions for the first and subsequent courses, respectively. In the same study, the LC and OS rates were 87.8% and 55.6%, respectively. Based on these data, the authors concluded that for patients with peripheral tumors and CP A liver function, repeated PBT is safe. More recently, researchers at PMRC reported the results of repeated PBT for 83 patients with HCC, specifically including patients who received 3 (*n* = 12) or 4 (*n* = 3) courses of PBT[53]. The median doses for the first, second, third, and fourth courses were 71.0, 70.0, 70.0, and 69.3 GyE, respectively, and the 5-year OS rate was 49.4%. Neither severe acute toxicity nor RILD was observed during the study period.

PBT FOR HCC WITH PORTAL VEIN TUMOR THROMBOSIS

Portal vain tumor thrombosis (PVTT) at the time of diagnosis is present in 30%-40% of all patients with advanced HCC[11], and PVTT is a poor prognostic factor with a median survival of 2.7 mo to 4.0 mo[54,55]. Currently, the Barcelona-Clinic Liver Cancer (BCLC) system recommends sorafenib as the only standard treatment in such patients[9]. However, the response rate to sorafenib ranges from 2% to 5%, and the median time to progression is only 2.8 mo[56]. Therefore, efficient local modalities leading to improve clinical outcomes are required. Unfortunately, no local modalities have been proven to be efficient for HCC with PVTT. Surgery and TACE alone are

not typically feasible because most cases of HCC with PVTT are unresectable, and the efficacy of TACE is limited due to the aortoportal shunt[57]. However, various studies have reported the outcomes of RT with or without TACE, with an objective response rate ranging from 40% to 60%. Furthermore, among responders, the median PFS is reported to be 15 mo to 20 mo[58-61].

The outcomes of PBT for HCC with PVTT have also been evaluated by PMRC. That study analyzed 12 HCC patients with PVTT in which a median dose regimen of 55 GyE in 10-22 fractions was used (range of 50 to 72 GyE in 10-22 fractions). Radiographic CR was achieved in 6 patients, and there was recanalization of the portal vein in 5 patients. Furthermore, the 2- and 5-year OS rates were 88% and 58%, respectively.

Lastly, the National Cancer Center of Korea reported on the outcomes of PBT using a simultaneous integrated boost technique for HCC with PVTT[62]. The dose regimens were determined depending on the distance of the tumor from GI OARs, and most tumors were treated with 50-66 GyE in 10 fractions while areas closer to an OAR were treated with 30 GyE in 10 fractions. The LC and OS rates reported in that study at 2 years were 88.1% and 51.1%, respectively. In addition, patients treated with equivalent dose of 2 Gy fractions for $\alpha/\beta = 10$ Gy (EQD2$_{10}$) of $\geqslant$ 80 GyE, tended to show better tumor vascular thrombosis response, LC, and OS.

PBT FOR LARGE HCCs

Tumor size is critical in determining treatment modalities in HCC[63]. Patients with large tumors are not candidates for ablation therapies or liver transplantation[64-66]. Although surgery seems to have role in the treatment of HCCs larger than 10 cm, few of these tumors are actual candidates for resection[67-69]. In such cases, RT represents an alternative modality for large tumors; however, it is not possible to avoid irradiating large volumes of normal liver tissues, which in turn limits sufficient dose escalation[70,71]. Importantly, PBT has the potential to overcome this limitation. Researchers at the University of Hokkaido performed dosimetric comparison of spot-scanning PBT *vs* IMRT for HCC[72]. For gross tumors of a nominal diameter more than 6.3 cm, the average risk of RILD according to the Lyman-normal-tissue complication probability model was estimated as 94.5% for IMRT compared to 6.2% for PBT. PMRC also reported the clinical result of PBT for 22 patients with HCC of size > 10 cm[73]. In that study, which employed a median dose regimen of 72.6 GyE in 22 fractions (range, 47.3 to 89.1 GyE in 10-35 fractions), the median tumor size was 11 cm (range, 10 cm to 14 cm). The LC and OS at 2 years were 87% and 36%, respectively. RILD occurred in 5 of 22 patients, 3 of whom showed CR.

PBT FOR HCC PATIENTS WITH POOR LIVER FUNCTION

HCC patients with cirrhotic livers or poor liver function

have a dismal prognosis with a median survival time of 3 to 9 mo[55]. Although poor liver function itself is a poor prognostic factor, there are limitations in applying local modalities to those patients because it is difficult to preserve the function of the residual untreated liver[74]. Researchers at PMRC reported on the clinical outcomes of PBT for 19 patients with HCC and CP C[75]. The median RT dose was 72 GyE in 16 fractions (range, 50 to 84 GyE in 10 to 24 fractions). The crude LC rate was 95% over a median follow-up of 17 mo, and the OS at 2 years was 42%. In addition, there were no therapy-related toxicities of grade $\geqslant$ 3 or deterioration of CP score. Among the total cohort, 14 patients exhibited an improved CP score. Researchers at PMRC also reported on the feasibility of PBT for HCC patients with uncontrollable ascites[76]. Specifically, they used PBT of 24 GyE in a single fraction, and performed precise adjustment of PBT immediately before irradiation. Although their studies only included three patients, objective responses were achieved for all the irradiated tumors, and there were no therapy-related toxicities higher than grade 3.

HEPATIC TOXICITY

RILD is seldom observed in the era of PBT for HCC, and there is very limited literature on PBT-related RILD. Researchers at PMRC evaluated 60 HCC patients treated with PBT consisting of 76 GyE in 20 fractions[77]. Seventy-eight percent of the patients had CP-A liver function, and 82% had liver cirrhosis; prior liver directed therapy had been performed in 60% of the patients. In that study, RILD was defined as anicteric ascites or asterixis. A total of 11 patients exhibited RILD, and seven patients died due to RILD. In addition, they reported that there were no cases of PBT-related RILD for patients with pretreatment indocyanine green retention at 15 min (ICG R15) less than 20%. On the other hand, RILD-related mortality of patients with a pretreatment ICG R15 over 50% was 75%. For patients with pretreatment ICG R15 of 20%-49%, V$_{30GyE}$ < 25% was identified as a predictor for PBT-related RILD. In more recent studies, changes in CP score were reported. Specifically, PMRC evaluated CP scores before and after PBT for 259 HCC patients[24], and found that among the 76% of patients with CP-A, 73% had cirrhosis of the liver and 63% had previously received liver directed treatment. In addition, the rates in the increase in point 1 CP score compared to baseline were 16% and 8% at 1 and 2 years, respectively. Likewise, the rates of point 2 from baseline CP sore were 11% and 22% at 1 and 2 years, respectively. On the other hand, literature from the US and South Korea indicate that only 4% of study patients have an increased CP score after PBT[25,43,78].

GT TRACT TOXICITY

The stomach, duodenum, and bowel are critical OARs because of their proximity to HCC tumors and the vulnerability of patients to radiation toxicity due to portal hypertension-induced gastroduodenopathy[79,80]. A group

of researchers from Loma Linda University reported that the incidence of GI toxicities ≥ grade 2 is 7% following RT[43]. Similarly, researchers at PMRC reported an incidence of ≥ grade 2 GI toxicities of 1%-2% in various populations[39,41,81]. For central tumors abutting the GI tract, however, the incidence of GI toxicities ≥ grade 2 increases to 8.5%, with all of the reported forms of GI toxicity involving hemorrhage[42]. Usually, PBT for HCC proximal to GI tract is prone to GI toxicity. Especially, GI tract located near the distal edge of SOBP is potentially vulnerable to severe toxicity because of the uncertainty of the proton beam range and the high RBE value at the distal edge and the fall off of the SOBP. Although there is no specific report regarding of the relation between the variation of RBE and the GI toxicities, several literatures reported the distal edge effects of PBT on toxicities of brain stem[82,83]. Therefore, special caution is necessary when the location of GI tract is near the distal edge of SOBP. Surgical insertion of tissue expander to displace GI tract away from target is considerable in that situation[84].

BILIARY AND OTHER GI TRACT TOXICITY

The biliary tract is an OAR that must be protected during liver RT. Indeed, several studies have reported symptomatic biliary inflammation with or without stricture in tumors related to the biliary system[85,86]. However, some studies have reported that RT for HCC can be safe with respect to biliary toxicity, even in tumors adjacent to the central biliary system[87,88]. For PBT in HCC, data on biliary complications is limited. Researchers at PMRC reviewed 162 patients who underwent PBT for HCC with a median RT dose of 72 GyE in 16 fractions[81]. Among the total cohort, three patients exhibited signs of biliary toxicity such as stenosis of the common bile duct (n = 1), and biloma with infection adjacent to the irradiated volume (n = 2).

CHEST WALL TOXICITY

As PBT allows for dose escalation in the treatment of HCC, the chest wall is frequently irradiated with increasing high doses. In tumors proximal to the chest wall, there is potential risk of chest wall-related toxicities[19]. Although PBT-related chest wall toxicity data are limited, there have been some published studies. Researchers at PMRC reported that the incidence of rib fracture is 16% after PBT of 66 GyE in 10 fractions in 67 HCC patients[89]. Specifically, the authors found that V60 at a biologically effective dose with α/β = 3 is the most statistically significant parameter for estimating the risk of rib fracture after hypofractionated PBT. In addition, researchers at the MD Anderson Cancer Center reported the chest wall toxicities of 135 patients with lung or hepatobiliary cancer who were treated with ≥ 52.5 GyE in 15 fractions of photon therapy or PBT[90]. Among the total cohort, 49 patients received PBT. During the median follow-up of 9

mo, 20 patients had grade 1 chest wall pain, while one patient had grade 2 chest wall pain. Furthermore, a chest wall V40 ≥ 150 cm^3 was identified as an independent predictor for chest wall toxicity. Researchers at the University of Washington and other institutions reported another set of data for chest wall toxicity, comprising 37 patients who underwent PBT with a median dose of 60 GyE (range, 35 to 67.5 GyE) in 15 fractions (range, 13 to 20 fractions)[91]. During a median follow-up of 11 mo, chest wall pain of grade ≥ 2 occurred in 30% of patients, with none of the patients exhibiting radiographic evidence of rib fracture. For a 15-fraction regimen, a V47 > 20 cm^3, V50 > 17 cm^3, and V58 > 8 cm^3 were associated with higher rates of chest wall toxicity. Although there is no consensus regarding dose constraints for chest wall toxicities, efforts should be made to reduce chest wall toxicities based on the reported various dose-volumetric parameters in PBT for HCC.

TECHNICAL ISSUES

Proton beams have unique physical properties including a finite range in the distal direction. This range is determined by the density of material in the beam path, and any perturbation in the densities can change the dosimetry of PBT more significantly than compared to traditional photon therapy[92]. This perturbation can be induced by internal organ motion, daily variation in body shape, uncertainties in machine settings, tissue inhomogeneity, and inaccuracies in dosing, ranges, and density calculations[93]. More specifically, the liver is vulnerable to uncertainties of PBT due to organ motion, the interface of air and soft tissue near the diaphragmatic dome, and variations in body shape due to ascites[76].

Various techniques have been investigated to manage the liver-specific problems associated with PBT. In order to overcome uncertainty due to organ motion, the PBT field should ideally encompass the entire range of motion of the tumor; however, this usually results in additional irradiation of normal tissues. Therefore, respiratory gating methods, abdominal compression, or breath hold techniques can be used to reduce the field such that OARs can be protected from unnecessary irradiation[94,95]. Because of the requirement for patient cooperation, identifying patients who are eligible for these techniques remains an important issue.

Techniques for motion control require fine image guidance. However, tumor and surrounding normal liver tissue are often undistinguishable on typical imaging studies such as orthogonal KV X-ray images or cone beam computed tomography[19]. To set a reference location for setup, radio-opaque fiducial markers are often utilized for more reliable and accurate image guidance[96]. Robust optimization is another method for managing imaging uncertainties. One reported algorithm incorporates the uncertainty directly into PBT optimization[97]. Using this algorithm, it is possible to quantify the quality of the treatment plan under certain geometric uncertainties, as

well as those from inaccurate calculations of range and tissue density.

FUTURE PERSPECTIVES

Several studies evaluating the role of PT for HCC are ongoing. These studies are aimed at comparing PBT with radiofrequency ablation, transarterial chemoembolization, or sorafenib, which are the current standard treatments recommended according to BCLC staging. The interim results of a randomized trial comparing PBT with transarterial chemoembolization were reported as discussed above[44]. In addition, various single-arm phase Ⅱ studies of PBT for HCC in specific clinical situations such as PVTT and inoperable disease are ongoing. The results of these studies will provide many answers to ongoing questions and increase the level of evidence in the field of PBT for HCC.

Technologies related to PBT are also under development. Almost all of the clinical data from PBT for HCC have relied on the passive scattering technique[20]. However, recently constructed PBT centers are now utilizing pencil beam scanning nozzles, which allow for manipulation of the intensity of proton therapy[98-100]. Pencil beam scanning shows better dose optimization and proximal edge conformity compared with passive scattering[101,102]. On the other hand, pencil beam scanning takes more time to deliver the proton beam, involves a broader lateral penumbra, and is more sensitive to organ motion[103-105]. Therefore, robustness optimization and evaluation tools, respiration management such as repainting, and fine image guidance are required for the use of pencil beam scanning in the treatment of HCC[106,107]. Although these issues continue to be investigated and studied, the pencil beam scanning method with simultaneous intensity boost technique is now being tried in HCC[108]. Nevertheless, in order to validate the effectiveness and safety of the pencil beam scanning PBT for HCC, large scale prospective trials will be necessary.

Biological issues are another field which can be more advanced in the future. If the RBE variation can be utilized in the optimization of treatment planning, the therapeutic window would be increased[33]. The variability of RBE is actively being investigated regarding the relevance of not only dose range but various value of α/β's from different tissues[109,110]. The biology of HCC is diverse according to the various genomic aberrations and the genetic association with radiosensitivity of HCC has been also investigated in *in vitro* and *in vivo*[111-113]. Although there is no current data for radiaosensitivity of HCC for PBT specifically, the investigations about the tissue specific radiosensitivity which can be represented with RBE will give the opportunity to provide the optimal strategy of tissue-specific PBT. This is the direction of the approach for PBT to be developed as precision medicine for HCC patients.

CONCLUSION

PBT has significant dosimetric advantages compared

with X-ray therapy, which has translated to significant differences in clinical outcomes for the treatment for HCC[20]. The American Society for Radiation Oncology includes HCC in the "group Ⅰ" indications for PBT, meaning that PBT is recognized as an effective and safe local modality for the treatment of HCC. Various studies comparing with other local modalities are ongoing, and we expect that PBT will become a mainstay of local treatment of HCC in the near future.

REFERENCES

1   **Ghouri YA**, Mian I, Rowe JH. Review of hepatocellular carcinoma: Epidemiology, etiology, and carcinogenesis. *J Carcinog* 2017; **16**: 1 [PMID: 28694740 DOI: 10.4103/jcar.JCar_9_16]

2   **Lafaro KJ**, Demirjian AN, Pawlik TM. Epidemiology of hepatocellular carcinoma. *Surg Oncol Clin N Am* 2015; **24**: 1-17 [PMID: 25444466 DOI: 10.1016/j.soc.2014.09.001]

3   **Mittal S**, El-Serag HB. Epidemiology of hepatocellular carcinoma: consider the population. *J Clin Gastroenterol* 2013; **47** Suppl: S2-S6 [PMID: 23632345 DOI: 10.1097/MCG.0b013e3182872f29]

4   **Fattovich G**, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology* 2004; **127**: S35-S50 [PMID: 15508101 DOI: 10.1053/j.gastro.2004.09.014]

5   **El-Serag HB**. Hepatocellular carcinoma. *N Engl J Med* 2011; **365**: 1118-1127 [PMID: 21992124 DOI: 10.1056/NEJMra1001683]

6   **Zhang DY**, Friedman SL. Fibrosis-dependent mechanisms of hepatocarcinogenesis. *Hepatology* 2012; **56**: 769-775 [PMID: 22378017 DOI: 10.1002/hep.25670]

7   **Toshikuni N**, Takuma Y, Tomokuni J, Yamamoto H. Planning Sonography Using Real-time Virtual Sonography and Contrast-enhanced Sonography for Radiofrequency Ablation of Inconspicuous Hepatocellular Carcinoma Nodules. *Hepatogastroenterology* 2015; **62**: 661-666 [PMID: 26897949]

8   **Zou Q**, Li J, Wu D, Yan Z, Wan X, Wang K, Shi L, Lau WY, Wu M, Shen F. Nomograms for Pre-operative and Post-operative Prediction of Long-Term Survival of Patients Who Underwent Repeat Hepatectomy for Recurrent Hepatocellular Carcinoma. *Ann Surg Oncol* 2016; **23**: 2618-2626 [PMID: 26903045 DOI: 10.1245/s10434-016-5136-0]

9   **Forner A**, Llovet JM, Bruix J. Hepatocellular carcinoma. *Lancet* 2012; **379**: 1245-1255 [PMID: 22353262 DOI: 10.1016/S0140-6736(11)61347-0]

10 **Sinn DH**, Gwak GY, Cho J, Paik SW, Yoo BC. Comparison of clinical manifestations and outcomes between hepatitis B virus- and hepatitis C virus-related hepatocellular carcinoma: analysis of a nationwide cohort. *PLoS One* 2014; **9**: e112184 [PMID: 25372403 DOI: 10.1371/journal.pone.0112184]

11 **Yu JI**, Park HC. Radiotherapy as valid modality for hepatocellular carcinoma with portal vein tumor thrombosis. *World J Gastroenterol* 2016; **22**: 6851-6863 [PMID: 27570422 DOI: 10.3748/wjg.v22.i30.6851]

12 **Dionisi F**, Ben-Josef E. The use of proton therapy in the treatment of gastrointestinal cancers: liver. *Cancer J* 2014; **20**: 371-377 [PMID: 25415681 DOI: 10.1097/PPO.0000000000000082]

13 **Kalogeridi MA**, Zygogianni A, Kyrgias G, Kouvaris J, Chatziioannou S, Kelekis N, Kouloulias V. Role of radiotherapy in the management of hepatocellular carcinoma: A systematic review. *World J Hepatol* 2015; **7**: 101-112 [PMID: 25625001 DOI: 10.4254/wjh.v7.i1.101]

14 **Klein J**, Dawson LA. Hepatocellular carcinoma radiation therapy: review of evidence and future opportunities. *Int J Radiat Oncol Biol Phys* 2013; **87**: 22-32 [PMID: 23219567 DOI: 10.1016/j.ijrobp.2012.08.043]

15 **Cho JY**, Paik YH, Park HC, Yu JI, Sohn W, Gwak GY, Choi MS, Lee JH, Koh KC, Paik SW, Yoo BC. The feasibility of combined transcatheter arterial chemoembolization and radiotherapy for advanced hepatocellular carcinoma. *Liver Int* 2014; **34**: 795-801 [PMID: 24350564 DOI: 10.1111/liv.12445]

16   **Kudo M**, Chung H, Osaki Y. Prognostic staging system for hepatocellular carcinoma (CLIP score): its value and limitations, and a proposal for a new staging system, the Japan Integrated Staging Score (JIS score). *J Gastroenterol* 2003; **38**: 207-215 [PMID: 12673442 DOI: 10.1007/s005350300038]

17   **Corbin KS**, Hellman S, Weichselbaum RR. Extracranial oligometastases: a subset of metastases curable with stereotactic radiotherapy. *J Clin Oncol* 2013; **31**: 1384-1390 [PMID: 23460715 DOI: 10.1200/JCO.2012.45.9651]

18   **Kim SW**, Oh D, Park HC, Lim DH, Shin SW, Cho SK, Gwak GY, Choi MS, Paik YH, Paik SW. Transcatheter arterial chemoembolization and radiation therapy for treatment-naïve patients with locally advanced hepatocellular carcinoma. *Radiat Oncol J* 2014; **32**: 14-22 [PMID: 24724047 DOI: 10.3857/roj.2014.32.1.14]

19   **Yeung RH**, Chapman TR, Bowen SR, Apisarnthanarax S. Proton beam therapy for hepatocellular carcinoma. *Expert Rev Anticancer Ther* 2017; **17**: 911-924 [PMID: 28825506 DOI: 10.1080/14737140.2017.1368392]

20   **Dionisi F**, Widesott L, Lorentini S, Amichetti M. Is there a role for proton therapy in the treatment of hepatocellular carcinoma? A systematic review. *Radiother Oncol* 2014; **111**: 1-10 [PMID: 24560761 DOI: 10.1016/j.radonc.2014.02.001]

21   **Wang X**, Krishnan S, Zhang X, Dong L, Briere T, Crane CH, Martel M, Gillin M, Mohan R, Beddar S. Proton radiotherapy for liver tumors: dosimetric advantages over photon plans. *Med Dosim* 2008; **33**: 259-267 [PMID: 18973852 DOI: 10.1016/j.meddos.2007.04.008]

22   **Gandhi SJ**, Liang X, Ding X, Zhu TC, Ben-Josef E, Plastaras JP, Metz JM, Both S, Apisarnthanarax S. Clinical decision tool for optimal delivery of liver stereotactic body radiation therapy: Photons versus protons. *Pract Radiat Oncol* 2015; **5**: 209-218 [PMID: 25703530 DOI: 10.1016/j.prro.2015.01.004]

23   **Lasley FD**, Mannina EM, Johnson CS, Perkins SM, Althouse S, Maluccio M, Kwo P, Cárdenes H. Treatment variables related to liver toxicity in patients with hepatocellular carcinoma, Child-Pugh class A and B enrolled in a phase 1-2 trial of stereotactic body radiation therapy. *Pract Radiat Oncol* 2015; **5**: e443-e449 [PMID: 25899219 DOI: 10.1016/j.prro.2015.02.007]

24   **Mizumoto M**, Okumura T, Hashimoto T, Fukuda K, Oshiro Y, Fukumitsu N, Abei M, Kawaguchi A, Hayashi Y, Ohkawa A, Hashii H, Kanemoto A, Moritake T, Tohno E, Tsuboi K, Sakae T, Sakurai H. Evaluation of liver function after proton beam therapy for hepatocellular carcinoma. *Int J Radiat Oncol Biol Phys* 2012; **82**: e529-e535 [PMID: 22284041 DOI: 10.1016/j.ijrobp.2011.05.056]

25   **Hong TS**, Wo JY, Yeap BY, Ben-Josef E, McDonnell EI, Blaszkowsky LS, Kwak EL, Allen JN, Clark JW, Goyal L, Murphy JE, Javle MM, Wolfgang JA, Drapek LC, Arellano RS, Mamon HJ, Mullen JT, Yoon SS, Tanabe KK, Ferrone CR, Ryan DP, DeLaney TF, Crane CH, Zhu AX. Multi-Institutional Phase II Study of High-Dose Hypofractionated Proton Beam Therapy in Patients With Localized, Unresectable Hepatocellular Carcinoma and Intrahepatic Cholangiocarcinoma. *J Clin Oncol* 2016; **34**: 460-468 [PMID: 26668346 DOI: 10.1200/JCO.2015.64.2710]

26   **Fukumitsu N**, Okumura T, Takizawa D, Makishima H, Numajiri H, Murofushi K, Ohnishi K, Mizumoto M, Aihara T, Ishikawa H, Tsuboi K, Sakurai H. Proton beam therapy for metastatic liver tumors. *Radiother Oncol* 2015; **117**: 322-327 [PMID: 26385268 DOI: 10.1016/j.radonc.2015.09.011]

27   **Engelsman M**, Schwarz M, Dong L. Physics controversies in proton therapy. *Semin Radiat Oncol* 2013; **23**: 88-96 [PMID: 23473685 DOI: 10.1016/j.semradonc.2012.11.003]

28   **Dawson LA**, Normolle D, Balter JM, McGinn CJ, Lawrence TS, Ten Haken RK. Analysis of radiation-induced liver disease using the Lyman NTCP model. *Int J Radiat Oncol Biol Phys* 2002; **53**: 810-821 [PMID: 12095546 DOI: 10.1016/S0360-3016(02)02846-8]

29   **Lawrence TS**, Robertson JM, Anscher MS, Jirtle RL, Ensminger WD, Fajardo LF. Hepatic toxicity resulting from cancer treatment. *Int J Radiat Oncol Biol Phys* 1995; **31**: 1237-1248 [PMID: 7713785 DOI: 10.1016/0360-3016(94)00418-K]

30   **Mizumoto M**, Okumura T, Hashimoto T, Fukuda K, Oshiro Y, Fukumitsu N, Abei M, Kawaguchi A, Hayashi Y, Ookawa A, Hashii H, Kanemoto A, Moritake T, Tohno E, Tsuboi K, Sakae T, Sakurai H. Proton beam therapy for hepatocellular carcinoma: a comparison of three treatment protocols. *Int J Radiat Oncol Biol Phys* 2011; **81**: 1039-1045 [PMID: 20888707 DOI: 10.1016/j.ijrobp.2010.07.015]

31   **Alan Mitteer R**, Wang Y, Shah J, Gordon S, Fager M, Butter PP, Jun Kim H, Guardiola-Salmeron C, Carabe-Fernandez A, Fan Y. Proton beam radiation induces DNA damage and cell apoptosis in glioma stem cells through reactive oxygen species. *Sci Rep* 2015; **5**: 13961 [PMID: 26354413 DOI: 10.1038/srep13961]

32   **Durante M**, Loeffler JS. Charged particles in radiation oncology. *Nat Rev Clin Oncol* 2010; **7**: 37-43 [PMID: 19949433 DOI: 10.1038/nrclinonc.2009.183]

33   **Paganetti H**. Relating proton treatments to photon treatments via the relative biological effectiveness-should we revise current clinical practice? *Int J Radiat Oncol Biol Phys* 2015; **91**: 892-894 [PMID: 25832682 DOI: 10.1016/j.ijrobp.2014.11.021]

34   **Matsumoto Y**, Matsuura T, Wada M, Egashira Y, Nishio T, Furusawa Y. Enhanced radiobiological effects at the distal end of a clinical proton beam: in vitro study. *J Radiat Res* 2014; **55**: 816-822 [PMID: 24824674 DOI: 10.1093/jrr/rrt230]

35   **Paganetti H**. Relative biological effectiveness (RBE) values for proton beam therapy. Variations as a function of biological endpoint, dose, and linear energy transfer. *Phys Med Biol* 2014; **59**: R419-R472 [PMID: 25361443 DOI: 10.1088/0031-9155/59/22/R419]

36   **Wouters BG**, Lam GK, Oelfke U, Gardey K, Durand RE, Skarsgard LD. Measurements of relative biological effectiveness of the 70 MeV proton beam at TRIUMF using Chinese hamster V79 cells and the high-precision cell sorter assay. *Radiat Res* 1996; **146**: 159-170 [PMID: 8693066 DOI: 10.2307/3579588]

37   **Carabe A**, Moteabbed M, Depauw N, Schuemann J, Paganetti H. Range uncertainty in proton therapy due to variable biological effectiveness. *Phys Med Biol* 2012; **57**: 1159-1172 [PMID: 22330133 DOI: 10.1088/0031-9155/57/5/1159]

38   **Paganetti H**, Niemierko A, Ancukiewicz M, Gerweck LE, Goitein M, Loeffler JS, Suit HD. Relative biological effectiveness (RBE) values for proton beam therapy. *Int J Radiat Oncol Biol Phys* 2002; **53**: 407-421 [PMID: 12023146 DOI: 10.1016/S0360-3016(02)02754-2]

39   **Mizumoto M**, Tokuuye K, Sugahara S, Nakayama H, Fukumitsu N, Ohara K, Abei M, Shoda J, Tohno E, Minami M. Proton beam therapy for hepatocellular carcinoma adjacent to the porta hepatis. *Int J Radiat Oncol Biol Phys* 2008; **71**: 462-467 [PMID: 18243571 DOI: 10.1016/j.ijrobp.2007.09.056]

40   **Fukumitsu N**, Sugahara S, Nakayama H, Fukuda K, Mizumoto M, Abei M, Shoda J, Thono E, Tsuboi K, Tokuuye K. A prospective study of hypofractionated proton beam therapy for patients with hepatocellular carcinoma. *Int J Radiat Oncol Biol Phys* 2009; **74**: 831-836 [PMID: 19304408 DOI: 10.1016/j.ijrobp.2008.10.073]

41   **Nakayama H**, Sugahara S, Tokita M, Fukuda K, Mizumoto M, Abei M, Shoda J, Sakurai H, Tsuboi K, Tokuuye K. Proton beam therapy for hepatocellular carcinoma: the University of Tsukuba experience. *Cancer* 2009; **115**: 5499-5506 [PMID: 19645024 DOI: 10.1002/cncr.24619]

42   **Nakayama H**, Sugahara S, Fukuda K, Abei M, Shoda J, Sakurai H, Tsuboi K, Matsuzaki Y, Tokuuye K. Proton beam therapy for hepatocellular carcinoma located adjacent to the alimentary tract. *Int J Radiat Oncol Biol Phys* 2011; **80**: 992-995 [PMID: 21543162 DOI: 10.1016/j.ijrobp.2010.03.015]

43   **Bush DA**, Kayali Z, Grove R, Slater JD. The safety and efficacy of high-dose proton beam radiotherapy for hepatocellular carcinoma: a phase 2 prospective trial. *Cancer* 2011; **117**: 3053-3059 [PMID: 21264826 DOI: 10.1002/cncr.25809]

44   **Bush DA**, Smith JC, Slater JD, Volk ML, Reeves ME, Cheng J, Grove R, de Vera ME. Randomized Clinical Trial Comparing Proton Beam Radiation Therapy with Transarterial Chemoembolization for Hepatocellular Carcinoma: Results of an Interim Analysis. *Int J Radiat Oncol Biol Phys* 2016; **95**: 477-482 [PMID: 27084661 DOI: 10.1016/j.ijrobp.2016.02.027]

45   **Kim TH**, Park JW, Kim YJ, Kim BH, Woo SM, Moon SH, Kim SS, Koh YH, Lee WJ, Park SJ, Kim JY, Kim DY, Kim CM. Phase I dose-escalation study of proton beam therapy for inoperable

hepatocellular carcinoma. *Cancer Res Treat* 2015; **47**: 34-45 [PMID: 25381830 DOI: 10.4143/crt.2013.218]

46 **Bae SH**, Park HC, Lim DH, Lee JA, Gwak GY, Choi MS, Lee JH, Koh KC, Paik SW, Yoo BC. Salvage treatment with hypofractionated radiotherapy in patients with recurrent small hepatocellular carcinoma. *Int J Radiat Oncol Biol Phys* 2012; **82**: e603-e607 [PMID: 22208963 DOI: 10.1016/j.ijrobp.2011.09.053]

47 **Park JH**, Yoon SM, Lim YS, Kim SY, Shim JH, Kim KM, Lee HC, Cho B, Park G, Kim JH. Two-week schedule of hypofractionated radiotherapy as a local salvage treatment for small hepatocellular carcinoma. *J Gastroenterol Hepatol* 2013; **28**: 1638-1642 [PMID: 23621519 DOI: 10.1111/jgh.12249]

48 **Lim DH**, Lee H, Park HC, Lee JA, Kim SW, Yoo BC, Koh KC, Lee JH, Choi MS, Gwak GY, Paik SW. The efficacy of high-dose 3-dimensional conformal radiation therapy in patients with small hepatocellular carcinoma not eligible for other local modalities. *Am J Clin Oncol* 2013; **36**: 162-166 [PMID: 22391433 DOI: 10.1097/COC.0b013e3182438dae]

49 **Abusaris H**, Hoogeman M, Nuyttens JJ. Re-irradiation: outcome, cumulative dose and toxicity in patients retreated with stereotactic radiotherapy in the abdominal or pelvic region. *Technol Cancer Res Treat* 2012; **11**: 591-597 [PMID: 22568625 DOI: 10.7785/tcrt.2012.500261]

50 **Lee DS**, Woo JY, Kim JW, Seong J. Re-Irradiation of Hepatocellular Carcinoma: Clinical Applicability of Deformable Image Registration. *Yonsei Med J* 2016; **57**: 41-49 [PMID: 26632381 DOI: 10.3349/ymj.2016.57.1.41]

51 **Seol SW**, Yu JI, Park HC, Lim DH, Oh D, Noh JM, Cho WK, Paik SW. Treatment outcome of hepatic re-irradiation in patients with hepatocellular carcinoma. *Radiat Oncol J* 2015; **33**: 276-283 [PMID: 26756027 DOI: 10.3857/roj.2015.33.4.276]

52 **Hashimoto T**, Tokuuye K, Fukumitsu N, Igaki H, Hata M, Kagei K, Sugahara S, Ohara K, Matsuzaki Y, Akine Y. Repeated proton beam therapy for hepatocellular carcinoma. *Int J Radiat Oncol Biol Phys* 2006; **65**: 196-202 [PMID: 16563656 DOI: 10.1016/j.ijrobp.2005.11.043]

53 **Oshiro Y**, Mizumoto M, Okumura T, Fukuda K, Fukumitsu N, Abei M, Ishikawa H, Takizawa D, Sakurai H. Analysis of repeated proton beam therapy for patients with hepatocellular carcinoma. *Radiother Oncol* 2017; **123**: 240-245 [PMID: 28366501 DOI: 10.1016/j.radonc.2017.03.004]

54 **Schöniger-Hekele M**, Müller C, Kutilek M, Oesterreicher C, Ferenci P, Gangl A. Hepatocellular carcinoma in Central Europe: prognostic features and survival. *Gut* 2001; **48**: 103-109 [PMID: 11115830 DOI: 10.1136/gut.48.1.103]

55 **Llovet JM**, Bustamante J, Castells A, Vilana R, Ayuso Mdel C, Sala M, Brú C, Rodés J, Bruix J. Natural history of untreated nonsurgical hepatocellular carcinoma: rationale for the design and evaluation of therapeutic trials. *Hepatology* 1999; **29**: 62-67 [PMID: 9862851 DOI: 10.1002/hep.510290145]

56 **Bruix J**, Raoul JL, Sherman M, Mazzaferro V, Bolondi L, Craxi A, Galle PR, Santoro A, Beaugrand M, Sangiovanni A, Porta C, Gerken G, Marrero JA, Nadel A, Shan M, Moscovici M, Voliotis D, Llovet JM. Efficacy and safety of sorafenib in patients with advanced hepatocellular carcinoma: subanalyses of a phase III trial. *J Hepatol* 2012; **57**: 821-829 [PMID: 22727733 DOI: 10.1016/j.jhep.2012.06.014]

57 **Lee HS**, Kim JS, Choi IJ, Chung JW, Park JH, Kim CY. The safety and efficacy of transcatheter arterial chemoembolization in the treatment of patients with hepatocellular carcinoma and main portal vein obstruction. A prospective controlled study. *Cancer* 1997; **79**: 2087-2094 [PMID: 9179054 DOI: 10.1002/(SICI)1097-0142(19970601)79:11<2087::AID-CNCR5>3.0.CO;2-M]

58 **Kim DY**, Park W, Lim DH, Lee JH, Yoo BC, Paik SW, Kho KC, Kim TH, Ahn YC, Huh SJ. Three-dimensional conformal radiotherapy for portal vein thrombosis of hepatocellular carcinoma. *Cancer* 2005; **103**: 2419-2426 [PMID: 15822130 DOI: 10.1002/cncr.21043]

59 **Yu JI**, Park HC, Lim DH, Park W, Yoo BC, Paik SW, Koh KC, Lee JH. Prognostic index for portal vein tumor thrombosis in patients with hepatocellular carcinoma treated with radiation therapy. *J Korean Med Sci* 2011; **26**: 1014-1022 [PMID: 21860551 DOI: 10.3346/jkms.2011.26.8.1014]

60 **Yu JI**, Yoon SM, Park HC, Kim JH, Kim TH, Park JW, Seong J, Lee IJ, Jang HS, Kay CS, Kim CY, Chie EK, Kim JH, Kim MS, Choi YM. Multicenter validation study of a prognostic index for portal vein tumor thrombosis in hepatocellular carcinoma. *Cancer Res Treat* 2014; **46**: 348-357 [PMID: 25036573 DOI: 10.4143/crt.2013.142]

61 **Yoon SM**, Lim YS, Won HJ, Kim JH, Kim KM, Lee HC, Chung YH, Lee YS, Lee SG, Park JH, Suh DJ. Radiotherapy plus transarterial chemoembolization for hepatocellular carcinoma invading the portal vein: long-term patient outcomes. *Int J Radiat Oncol Biol Phys* 2012; **82**: 2004-2011 [PMID: 21621346 DOI: 10.1016/j.ijrobp.2011.03.019]

62 **Kim DY**, Park JW, Kim TH, Kim BH, Moon SH, Kim SS, Woo SM, Koh YH, Lee WJ, Kim CM. Risk-adapted simultaneous integrated boost-proton beam therapy (SIB-PBT) for advanced hepatocellular carcinoma with tumour vascular thrombosis. *Radiother Oncol* 2017; **122**: 122-129 [PMID: 28034460 DOI: 10.1016/j.radonc.2016.12.014]

63 **Bruix J**, Sherman M; Practice Guidelines Committee, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma. *Hepatology* 2005; **42**: 1208-1236 [PMID: 16250051 DOI: 10.1002/hep.20933]

64 **Vilana R**, Bruix J, Bru C, Ayuso C, Solé M, Rodés J. Tumor size determines the efficacy of percutaneous ethanol injection for the treatment of small hepatocellular carcinoma. *Hepatology* 1992; **16**: 353-357 [PMID: 1322349 DOI: 10.1002/hep.1840160212]

65 **Livraghi T**, Goldberg SN, Lazzaroni S, Meloni F, Ierace T, Solbiati L, Gazelle GS. Hepatocellular carcinoma: radio-frequency ablation of medium and large lesions. *Radiology* 2000; **214**: 761-768 [PMID: 10715043 DOI: 10.1148/radiology.214.3.r00mr02761]

66 **Haug CE**, Jenkins RL, Rohrer RJ, Auchincloss H, Delmonico FL, Freeman RB, Lewis WD, Cosimi AB. Liver transplantation for primary hepatic cancer. *Transplantation* 1992; **53**: 376-382 [PMID: 1310823 DOI: 10.1097/00007890-199202010-00021]

67 **Noguchi T**, Kawarada Y, Kitagawa M, Ito F, Sakurai H, Machishi H, Yamagiwa K, Yokoi H, Mizumoto R. Clinicopathologic factors influencing the long-term prognosis following hepatic resection for large hepatocellular carcinoma more than 10 cm in diameter. *Semin Oncol* 1997; **24**: S6-7-S6-13 [PMID: 9151910]

68 **Poon RT**, Fan ST, Wong J. Selection criteria for hepatic resection in patients with large hepatocellular carcinoma larger than 10 cm in diameter. *J Am Coll Surg* 2002; **194**: 592-602 [PMID: 12022599 DOI: 10.1016/S1072-7515(02)01163-8]

69 **Pandey D**, Lee KH, Wai CT, Wagholikar G, Tan KC. Long term outcome and prognostic factors for large hepatocellular carcinoma (10 cm or more) after surgical resection. *Ann Surg Oncol* 2007; **14**: 2817-2823 [PMID: 17690940 DOI: 10.1245/s10434-007-9518-1]

70 **McGinn CJ**, Ten Haken RK, Ensminger WD, Walker S, Wang S, Lawrence TS. Treatment of intrahepatic cancers with radiation doses based on a normal tissue complication probability model. *J Clin Oncol* 1998; **16**: 2246-2252 [PMID: 9626227 DOI: 10.1200/JCO.1998.16.6.2246]

71 **Hawkins MA**, Dawson LA. Radiation therapy for hepatocellular carcinoma: from palliation to cure. *Cancer* 2006; **106**: 1653-1663 [PMID: 16541431 DOI: 10.1002/cncr.21811]

72 **Toramatsu C**, Katoh N, Shimizu S, Nihongi H, Matsuura T, Takao S, Miyamoto N, Suzuki R, Sutherland K, Kinoshita R, Onimaru R, Ishikawa M, Umegaki K, Shirato H. What is the appropriate size criterion for proton radiotherapy for hepatocellular carcinoma? A dosimetric comparison of spot-scanning proton therapy versus intensity-modulated radiation therapy. *Radiat Oncol* 2013; **8**: 48 [PMID: 23497543 DOI: 10.1186/1748-717X-8-48]

73 **Sugahara S**, Oshiro Y, Nakayama H, Fukuda K, Mizumoto M, Abei M, Shoda J, Matsuzaki Y, Thono E, Tokita M, Tsuboi K, Tokuuye K. Proton beam therapy for large hepatocellular carcinoma. *Int J Radiat Oncol Biol Phys* 2010; **76**: 460-466 [PMID: 19427743 DOI: 10.1016/j.ijrobp.2009.02.030]

74 **Ohara K**, Okumura T, Tsuji H, Chiba T, Min M, Tatsuzaki H, Tsujii H, Akine Y, Itai Y. Radiation tolerance of cirrhotic livers in

relation to the preserved functional capacity: analysis of patients with hepatocellular carcinoma treated by focused proton beam radiotherapy. *Int J Radiat Oncol Biol Phys* 1997; **38**: 367-372 [PMID: 9226325 DOI: 10.1016/S0360-3016(96)00587-1]

75   **Hata M**, Tokuuye K, Sugahara S, Fukumitsu N, Hashimoto T, Ohnishi K, Nemoto K, Ohara K, Matsuzaki Y, Akine Y. Proton beam therapy for hepatocellular carcinoma patients with severe cirrhosis. *Strahlenther Onkol* 2006; **182**: 713-720 [PMID: 17149578 DOI: 10.1007/s00066-006-1564-2]

76   **Hata M**, Tokuuye K, Sugahara S, Tohno E, Fukumitsu N, Hashimoto T, Ohnishi K, Nemoto K, Ohara K, Sakae T, Akine Y. Proton irradiation in a single fraction for hepatocellular carcinoma patients with uncontrollable ascites. Technical considerations and results. *Strahlenther Onkol* 2007; **183**: 411-416 [PMID: 17680219 DOI: 10.1007/s00066-007-1640-2]

77   **Kawashima M**, Kohno R, Nakachi K, Nishio T, Mitsunaga S, Ikeda M, Konishi M, Takahashi S, Gotohda N, Arahira S, Zenda S, Ogino T, Kinoshita T. Dose-volume histogram analysis of the safety of proton beam therapy for unresectable hepatocellular carcinoma. *Int J Radiat Oncol Biol Phys* 2011; **79**: 1479-1486 [PMID: 20605350 DOI: 10.1016/j.ijrobp.2009.12.048]

78   **Kim JW**, Seong J, Lee IJ, Woo JY, Han KH. Phase I dose escalation study of helical intensity-modulated radiotherapy-based stereotactic body radiotherapy for hepatocellular carcinoma. *Oncotarget* 2016; **7**: 40756-40766 [PMID: 27213593 DOI: 10.18632/oncotarget.9450]

79   **Chen LS**, Lin HC, Hwang SJ, Lee FY, Hou MC, Lee SD. Prevalence of gastric ulcer in cirrhotic patients and its relation to portal hypertension. *J Gastroenterol Hepatol* 1996; **11**: 59-64 [PMID: 8672743 DOI: 10.1111/j.1440-1746.1996.tb00011.x]

80   **Kitano S**, Dolgor B. Does portal hypertension contribute to the pathogenesis of gastric ulcer associated with liver cirrhosis? *J Gastroenterol* 2000; **35**: 79-86 [PMID: 10680661 DOI: 10.1007/s005350050018]

81   **Chiba T**, Tokuuye K, Matsuzaki Y, Sugahara S, Chuganji Y, Kagei K, Shoda J, Hata M, Abei M, Igaki H, Tanaka N, Akine Y. Proton beam therapy for hepatocellular carcinoma: a retrospective review of 162 patients. *Clin Cancer Res* 2005; **11**: 3799-3805 [PMID: 15897579 DOI: 10.1158/1078-0432.CCR-04-1350]

82   **Indelicato DJ**, Flampouri S, Rotondo RL, Bradley JA, Morris CG, Aldana PR, Sandler E, Mendenhall NP. Incidence and dosimetric parameters of pediatric brainstem toxicity following proton therapy. *Acta Oncol* 2014; **53**: 1298-1304 [PMID: 25279957 DOI: 10.3109/0284186X.2014.957414]

83   **Giantsoudi D**, Sethi RV, Yeap BY, Eaton BR, Ebb DH, Caruso PA, Rapalino O, Chen YL, Adams JA, Yock TI, Tarbell NJ, Paganetti H, MacDonald SM. Incidence of CNS Injury for a Cohort of 111 Patients Treated With Proton Therapy for Medulloblastoma: LET and RBE Associations for Areas of Injury. *Int J Radiat Oncol Biol Phys* 2016; **95**: 287-296 [PMID: 26691786 DOI: 10.1016/j.ijrobp.2015.09.015]

84   **Komatsu S**, Hori Y, Fukumoto T, Murakami M, Hishikawa Y, Ku Y. Surgical spacer placement and proton radiotherapy for unresectable hepatocellular carcinoma. *World J Gastroenterol* 2010; **16**: 1800-1803 [PMID: 20380016 DOI: 10.3748/wjg.v16.i14.1800]

85   **Cherqui D**, Palazzo L, Piedbois P, Charlotte F, Duvoux C, Duron JJ, Fagniez PL, Valla D. Common bile duct stricture as a late complication of upper abdominal radiotherapy. *J Hepatol* 1994; **20**: 693-697 [PMID: 7930466 DOI: 10.1016/S0168-8278(05)80136-9]

86   **Halevy A**, Adam A, Stamp G, Benjamin IS, Blumgart LH. Radiation stricture of the biliary ducts: an emerging new entity? *HPB Surg* 1992; **5**: 267-270 [PMID: 1390364 DOI: 10.1155/1992/52618]

87   **Eriguchi T**, Takeda A, Sanuki N, Oku Y, Aoki Y, Shigematsu N, Kunieda E. Acceptable toxicity after stereotactic body radiation therapy for liver tumors adjacent to the central biliary system. *Int J Radiat Oncol Biol Phys* 2013; **85**: 1006-1011 [PMID: 23102838 DOI: 10.1016/j.ijrobp.2012.09.012]

88   **Yu JI**, Park HC, Lim DH, Paik SW. Do Biliary Complications after Hypofractionated Radiation Therapy in Hepatocellular Carcinoma Matter? *Cancer Res Treat* 2016; **48**: 574-582 [PMID: 26194367 DOI: 10.4143/crt.2015.076]

89   **Kanemoto A**, Mizumoto M, Okumura T, Takahashi H, Hashimoto T, Oshiro Y, Fukumitsu N, Moritake T, Tsuboi K, Sakae T, Sakurai H. Dose-volume histogram analysis for risk factors of radiation-induced rib fracture after hypofractionated proton beam therapy for hepatocellular carcinoma. *Acta Oncol* 2013; **52**: 538-544 [PMID: 22950386 DOI: 10.3109/0284186X.2012.718094]

90   **Swanick CW**, Allen PK, Tao R, Olanrewaju AM, Sutton JR, Lin SH, Welsh J, Das P, Chang JY, Crane CH, Gomez DR. Incidence and predictors of chest wall toxicity after high-dose radiation therapy in 15 fractions. *Pract Radiat Oncol* 2017; **7**: 63-71 [PMID: 27637136 DOI: 10.1016/j.prro.2016.05.009]

91   **Yeung R**, Bowen SR, Chapman TR, MacLennan GT, Apisarnthanarax S. Chest wall toxicity after hypofractionated proton beam therapy for liver malignancies. *Pract Radiat Oncol* 2018; **8**: 287-293 [PMID: 29452863 DOI: 10.1016/j.prro.2017.12.007]

92   **Fukumitsu N**, Hashimoto T, Okumura T, Mizumoto M, Tohno E, Fukuda K, Abei M, Sakae T, Sakurai H. Investigation of the geometric accuracy of proton beam irradiation in the liver. *Int J Radiat Oncol Biol Phys* 2012; **82**: 826-833 [PMID: 21236603 DOI: 10.1016/j.ijrobp.2010.10.028]

93   **Zhang Y**, Huth I, Weber DC, Lomax AJ. A statistical comparison of motion mitigation performances and robustness of various pencil beam scanned proton systems for liver tumour treatments. *Radiother Oncol* 2018; [PMID: 29459153 DOI: 10.1016/j.radonc.2018.01.019]

94   **Hong TS**, DeLaney TF, Mamon HJ, Willett CG, Yeap BY, Niemierko A, Wolfgang JA, Lu HM, Adams J, Weyman EA, Arellano RS, Blaszkowsky LS, Allen JN, Tanabe KK, Ryan DP, Zhu AX. A prospective feasibility study of respiratory-gated proton beam therapy for liver tumors. *Pract Radiat Oncol* 2014; **4**: 316-322 [PMID: 25194100 DOI: 10.1016/j.prro.2013.10.002]

95   **Yu JI**, Yoo GS, Cho S, Jung SH, Han Y, Park S, Lee B, Kang W, Sinn DH, Paik YH, Gwak GY, Choi MS, Lee JH, Koh KC, Paik SW, Park HC. Initial clinical outcomes of proton beam radiotherapy for hepatocellular carcinoma. *Radiat Oncol J* 2018; **36**: 25-34 [PMID: 29580046 DOI: 10.3857/roj.2017.00409]

96   **Habermehl D**, Henkner K, Ecker S, Jäkel O, Debus J, Combs SE. Evaluation of different fiducial markers for image-guided radiotherapy and particle therapy. *J Radiat Res* 2013; **54** Suppl 1: i61-i68 [PMID: 23824129 DOI: 10.1093/jrr/rrt071]

97   **McGowan SE**, Burnet NG, Lomax AJ. Treatment planning optimisation in proton therapy. *Br J Radiol* 2013; **86**: 20120288 [PMID: 23255545 DOI: 10.1259/bjr.20120288]

98   **Lomax AJ**, Boehringer T, Coray A, Egger E, Goitein G, Grossmann M, Juelke P, Lin S, Pedroni E, Rohrer B, Roser W, Rossi B, Siegenthaler B, Stadelmann O, Stauble H, Vetter C, Wisser L. Intensity modulated proton therapy: a clinical example. *Med Phys* 2001; **28**: 317-324 [PMID: 11318312 DOI: 10.1118/1.1350587]

99   **Chung K**, Han Y, Kim J, Ahn SH, Ju SG, Jung SH, Chung Y, Cho S, Jo K, Shin EH, Hong CS, Shin JS, Park S, Kim DH, Kim HY, Lee B, Shibagaki G, Nonaka H, Sasai K, Koyabu Y, Choi C, Huh SJ, Ahn YC, Pyo HR, Lim DH, Park HC, Park W, Oh DR, Noh JM, Yu JI, Song S, Lee JE, Lee B, Choi DH. The first private-hospital based proton therapy center in Korea; status of the Proton Therapy Center at Samsung Medical Center. *Radiat Oncol J* 2015; **33**: 337-343 [PMID: 26756034 DOI: 10.3857/roj.2015.33.4.337]

100 **Smith A**, Gillin M, Bues M, Zhu XR, Suzuki K, Mohan R, Woo S, Lee A, Komaki R, Cox J, Hiramoto K, Akiyama H, Ishida T, Sasaki T, Matsuda K. The M. D. Anderson proton therapy system. *Med Phys* 2009; **36**: 4068-4083 [PMID: 19810479 DOI: 10.1118/1.3187229]

101 **Lomax AJ**, Pedroni E, Rutz H, Goitein G. The clinical potential of intensity modulated proton therapy. *Z Med Phys* 2004; **14**: 147-152 [PMID: 15462415 DOI: 10.1078/0939-3889-00217]

102 **Giantsoudi D**, Grassberger C, Craft D, Niemierko A, Trofimov A, Paganetti H. Linear energy transfer-guided optimization in intensity modulated proton therapy: feasibility study and clinical potential. *Int J Radiat Oncol Biol Phys* 2013; **87**: 216-222 [PMID: 23790771 DOI: 10.1016/j.ijrobp.2013.05.013]

103 **Kaderka R**, Schardt D, Durante M, Berger T, Ramm U, Licher J, La Tessa C. Out-of-field dose measurements in a water phantom

using different radiotherapy modalities. *Phys Med Biol* 2012; **57**: 5059-5074 [PMID: 22836598 DOI: 10.1088/0031-9155/57/16/5059]

104 **Fava G**, Widesott L, Fellin F, Amichetti M, Viesi V, Lomax AJ, Lederer L, Hug EB, Fiorino C, Salvadori G, Di Muzio N, Schwarz M. In-gantry or remote patient positioning? Monte Carlo simulations for proton therapy centers of different sizes. *Radiother Oncol* 2012; **103**: 18-24 [PMID: 22119372 DOI: 10.1016/j.radonc.2011.11.004]

105 **Park PC**, Zhu XR, Lee AK, Sahoo N, Melancon AD, Zhang L, Dong L. A beam-specific planning target volume (PTV) design for proton therapy to account for setup and range uncertainties. *Int J Radiat Oncol Biol Phys* 2012; **82**: e329-e336 [PMID: 21703781 DOI: 10.1016/j.ijrobp.2011.05.011]

106 **Unkelbach J**, Chan TC, Bortfeld T. Accounting for range uncertainties in the optimization of intensity modulated proton therapy. *Phys Med Biol* 2007; **52**: 2755-2773 [PMID: 17473350 DOI: 10.1088/0031-9155/52/10/009]

107 **Knopf AC**, Hong TS, Lomax A. Scanned proton radiotherapy for mobile targets-the effectiveness of re-scanning in the context of different treatment planning approaches and for different motion characteristics. *Phys Med Biol* 2011; **56**: 7257-7271 [PMID: 22037710 DOI: 10.1088/0031-9155/56/22/016]

108 **Apisarnthanarax S**, Saini J, O'Ryan-Blair A, Castro J, Bowen SR. Intensity Modulated Proton Therapy with Advanced Planning Techniques in a Challenging Hepatocellular Carcinoma Patient. *Cureus* 2017; **9**: e1674 [PMID: 29152431 DOI: 10.7759/cureus.1674]

109 **Hojo H**, Dohmae T, Hotta K, Kohno R, Motegi A, Yagishita A, Makinoshima H, Tsuchihara K, Akimoto T. Difference in the relative biological effectiveness and DNA damage repair processes in response to proton beam therapy according to the positions of the spread out Bragg peak. *Radiat Oncol* 2017; **12**: 111 [PMID: 28673358 DOI: 10.1186/s13014-017-0849-1]

110 **Ilicic K**, Combs SE, Schmid TE. New insights in the relative radiobiological effectiveness of proton irradiation. *Radiat Oncol* 2018; **13**: 6 [PMID: 29338744 DOI: 10.1186/s13014-018-0954-9]

111 **Khemlina G**, Ikeda S, Kurzrock R. The biology of Hepatocellular carcinoma: implications for genomic and immune therapies. *Mol Cancer* 2017; **16**: 149 [PMID: 28854942 DOI: 10.1186/s12943-017-0712-x]

112 **Jin Q**, Li XJ, Cao PG. miR-26b enhances radiosensitivity of hepatocellular carcinoma cells by targeting EphA2. *Iran J Basic Med Sci* 2016; **19**: 851-857 [PMID: 27746866]

113 **Yu L**, Sun Y, Li J, Wang Y, Zhu Y, Shi Y, Fan X, Zhou J, Bao Y, Xiao J, Cao K, Cao P. Silencing the Girdin gene enhances radio-sensitivity of hepatocellular carcinoma via suppression of glycolytic metabolism. *J Exp Clin Cancer Res* 2017; **36**: 110 [PMID: 28810896 DOI: 10.1186/s13046-017-0580-7]

Encapsulating peritoneal sclerosis

Christopher J Danford, Steven C Lin, Martin P Smith, Jacqueline L Wolf

Christopher J Danford, Steven C Lin, Jacqueline L Wolf, Division of Gastroenterology and Hepatology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115, United States

Martin P Smith, Department of Radiology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115, United States

ORCID number: Christopher J Danford (0000-0003-0322-7705); Steven C Lin (0000-0003-4620-1505); Martin P Smith (0000-0003-0775-6516); Jacqueline L Wolf (0000-0002-7748-394X).

Abstract

Encapsulating peritoneal sclerosis (EPS) is a debilitating condition characterized by a fibrocollagenous membrane encasing the small intestine, resulting in recurrent small bowel obstructions. EPS is most commonly associated with long-term peritoneal dialysis, though medications, peritoneal infection, and systemic inflammatory disorders have been implicated. Many cases remain idiopathic. Diagnosis is often delayed given the rarity of the disorder combined with non-specific symptoms and laboratory findings. Although cross-sectional imaging with computed tomography of the abdomen can be suggestive of the disorder, many patients undergo exploratory laparotomy for diagnosis. Mortality approaches 50% one year after diagnosis. Treatment for EPS involves treating the underlying condition or eliminating possible inciting agents (*i.e.* peritoneal dialysis, medications, infections) and nutritional support, frequently with total parenteral nutrition. EPS-specific treatment depends on the disease stage. In the inflammatory stage, corticosteroids are the treatment of choice, while in the fibrotic stage, tamoxifen may be beneficial. In practice, distinguishing between stages may be difficult and both may be used. Surgical intervention, consisting of peritonectomy and enterolysis, is time-consuming and high-risk and is reserved for situations in which conservative medical therapy fails in institutions with surgical expertise in this area. Herein we review the available literature of the etiology, pathogenesis, diagnosis, and treatment of this rare, but potentially devastating disease.

Key words: Abdominal cocoon; Sclerosing encapsulating peritonitis; Peritoneal sclerosis; Peritonectomy; Enterolysis; Peritoneal dialysis; Tamoxifen; Corticosteroids

Core tip: Encapsulating peritoneal sclerosis (EPS) is a rare, but potentially devastating disorder. Most literature is derived from the nephrology literature surrounding peritoneal dialysis, however, the gastroenterologist is likely to encounter EPS from a variety of etiologies. We present a comprehensive review of EPS from all etiologies and a summary of treatments from a gastroenterologist's perspective including the role of nutrition, surgery, immunosuppression, anti-fibrotic agents, and the novel use of μ-opioid antagonists and guanylate cyclase C agonists in the management of patients with EPS.

INTRODUCTION

Definition

Encapsulating peritoneal sclerosis (EPS) is a rare clinical syndrome characterized by an acquired, inflammatory fibrocollagenous membrane encasing the small intestine, resulting in symptoms of bowel obstruction. It is defined by the International Society for Peritoneal Dialysis as "a syndrome continuously, intermittently, or repeatedly presenting with symptoms of intestinal obstruction caused by adhesions of a diffusely thickened peritoneum"[1]. Owtschinnikow first described encasement of the intestines by a fibrocollagenous membrane in 1907 and coined the term *peritonitis chronica fibrosa incapsulata*[2]. EPS has also been known as *abdominal cocoon* and *sclerosing encapsulating peritonitis*. It should not be confused with peritoneal encapsulation, a congenital condition characterized by an accessory peritoneal membrane encapsulating the intestines, without adhesions to the encased intestine often incidentally recognized during unrelated surgery[3].

Etiology

EPS can be divided into primary (idiopathic) or secondary in which a trigger for the inflammatory process can be identified. Primary EPS was classically thought to afflict adolescent women in tropical and subtropical areas leading to theories of retrograde menstruation or gynecologic infection as the cause. Though the largest studies of primary EPS confirm its equatorial predilection, men are more commonly affected than women in a 2:1 ratio[4,5] and the etiology remains unclear.

In secondary EPS, a local or systemic factor can be identified as triggering peritoneal inflammation. Implicated triggers include medications[6-9], infection[10-16], mechanical or chemical intraperitoneal irritants[17-27], cirrhosis[28], organ transplantation[29-31], endometriosis[32], gynecologic neoplasms[33,34], dermoid cyst rupture[35], and systemic rheumatologic and inflammatory disorders[36-38] (Table 1).

Pathophysiology

EPS is thought to occur when a peritoneal inflammatory process (inciting factor) occurs in patients with a predisposing condition (Figure 1). In peritoneal dialysis (PD) literature, this is referred to as the "two-hit" hypothesis[39] in which the "first hit" or predisposing condition is the non-inflammatory peritoneal sclerosis resulting from repeated dialysis sessions. In support of this, cumulative incidence of EPS increases dramatically with time on PD[40,41]. A proinflammatory "second hit"[42] precipitates a cascade of proinflammatory [transforming growth factor β1 (TGFβ1), interleukin-6 (IL-6), CCN2] and proangiogenic [vascular endothelial growth factor (VEGF)] cytokines[43,44]. TGFβ1 promotes transdifferentiation of peritoneal mesothelial to mesenchymal cells resulting in mesothelial cell depletion[45,46], increased production of extracellular matrix components [collagen type 1, alpha 1 (COL1A1)] and fibrogenesis resulting in a fibrocollagenous cocoon[47] (Figure 1).

In PD, genetic variation in the receptor for advanced glycation end products may predispose an individual to peritoneal deterioration[48]. While no genetic studies exist outside the PD literature, genetic predisposition may explain why only a small proportion of patients with recurrent peritonitis develop EPS or why an individual may develop EPS even with a single, discrete exposure[24]. Among gynecologic neoplasms, reports of EPS are confined to luteinizing neoplasms[33,34] without other overt inflammatory trigger, leading to the hypothesis that they directly stimulate peritoneal inflammation and fibrosis, though the potential stimulatory cytokine has yet to be identified[49].
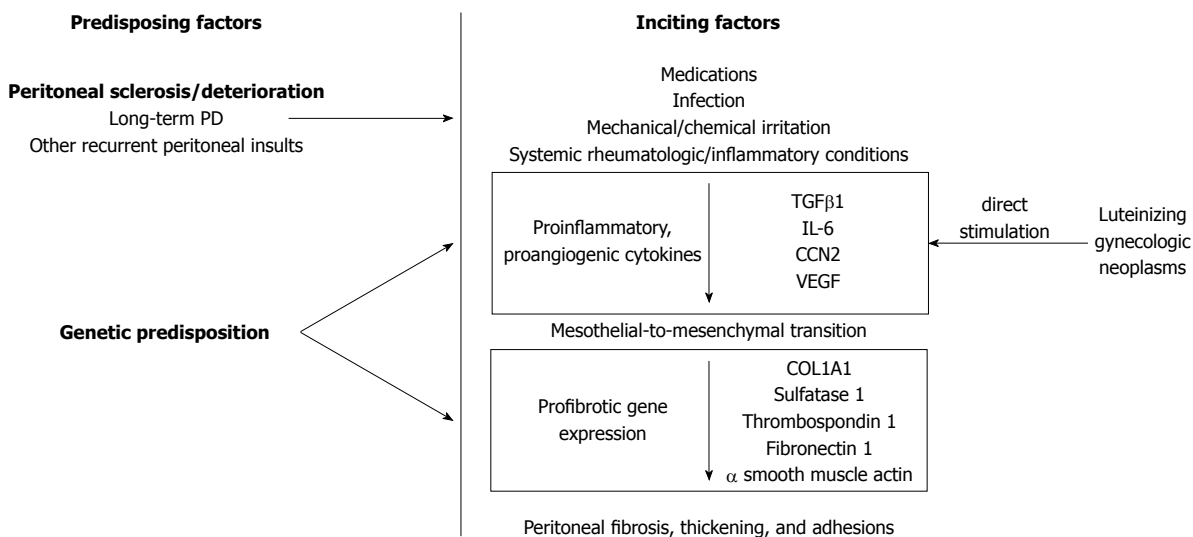
Epidemiology and natural history

Given its rarity and heterogeneity of etiologies, the incidence and prevalence of EPS as a whole is unknown. In a review of idiopathic EPS, cases were more commonly reported from tropical and subtropical countries [China (54%), India (18%), Turkey (9%), and Nigeria (3%)][6]. The mean age was 34.7 (range 7-87 years) with a 2:1 male predominance.

In peritoneal dialysis, the annual incidence of EPS varies from 0.14% to 2.5%[41,50] with decreasing incidence in more recent studies likely due to improved dialysis techniques[17,50]. The most significant risk factor for EPS development is duration of PD[17] with a low cumulative incidence at 3 years increasing after 5 years[17,40,41]. At 8 years, 10%-20% of PD patients will develop EPS[17,51-54]. The prevalence of EPS in PD has been observed between 0.4% and 8.9%[17]. Mortality in PD patients approaches 50% one year after diagnosis[17,50,55] though it is difficult to ascertain how much of this is related to EPS or the comorbidities that accompany end-stage renal disease.

Table 1 Classification and etiologies of encapsulating peritoneal sclerosis

Primary (idiopathic)	
2:1 (male:female)	
Most commonly reported in tropical/subtropical regions	
Secondary	
Medications	Mechanical or chemical irritation
Practolol[6]	Peritoneal dialysis[17]
Methotrexate[7,8]	Intraperitoneal chemotherapy[18]
Antiepileptic drugs[9]	Ventriculoperitoneal shunt[20]
Infection	Peritoneovenous shunt[21]
Tuberculosis[10]	Intraperitoneal iodine[22]
Non-tuberculous mycobacteria[11]	Abdominal trauma[23]
Bacterial peritonitis[12]	Intraabdominal surgery[24]
Cytomegalovirus[13]	Foreign body[25]
Fungus[14,15]	Talcum powder[19]
Parasite[16]	Asbestos[26]
Cirrhosis[28]	Silica[27]
Organ transplantation	Endometriosis[32]
Liver[29]	Dermoid cyst rupture[35]
Small intestine[30]	Rheumatologic/systemic inflammatory
Renal[31]	conditions
Gynecologic neoplasms	Sarcoidosis[36]
Luteinized thecoma[33]	Systemic lupus erythematosis[37]
Luteinizing granulosa cell tumor[34]	Familial Mediterranean fever[38]

Figure 1 Pathophysiology of encapsulating peritoneal sclerosis. Certain predisposing factors may be present for encapsulating peritoneal sclerosis to form, such as genetic predisposition or being on long-term peritoneal dialysis. Inciting factors that may push the physiology towards a pro-inflammatory and pro-fibrotic state include medications, repeated chemical or mechanical peritoneal irritation, recurrent infections, and systemic rheumatologic or inflammatory conditions.

DIAGNOSIS

Clinical presentation

The diagnosis of EPS is clinical, based on a constellation of clinical findings, and confirmed radiographically or by laparotomy[5,17,56,57]. No gold standard exists for diagnosis. However, a diagnostic algorithm is proposed in Figure 2. A majority of PD-associated EPS patients present after withdrawal of PD, with onset of symptoms occurring up to 5 years later[17]. In the largest case series of idiopathic EPS, the average duration of symptoms was 3.9 years prior to presentation with the vast majority presenting malnourished (75%, mean BMI 17.5 kg/m^2)[4]. The most common presenting symptoms were abdominal

pain (86%), abdominal distension (82%), and nausea and vomiting (54%). Twenty-nine percent of patients underwent "emergency surgery" on presentation implying that, despite the chronic, insidious nature of the disease, a significant percentage present with more acute obstruction, ischemia, or perforation[4].

Imaging

Imaging is often helpful in differentiating EPS from other causes of intestinal obstruction. Abdominal plain films showing peritoneal calcification as well as dilated loops of bowel with air-fluid levels may suggest advanced EPS[56,58]. Small bowel follow-through may show delayed transit, distension proximal to small bowel adhesions,
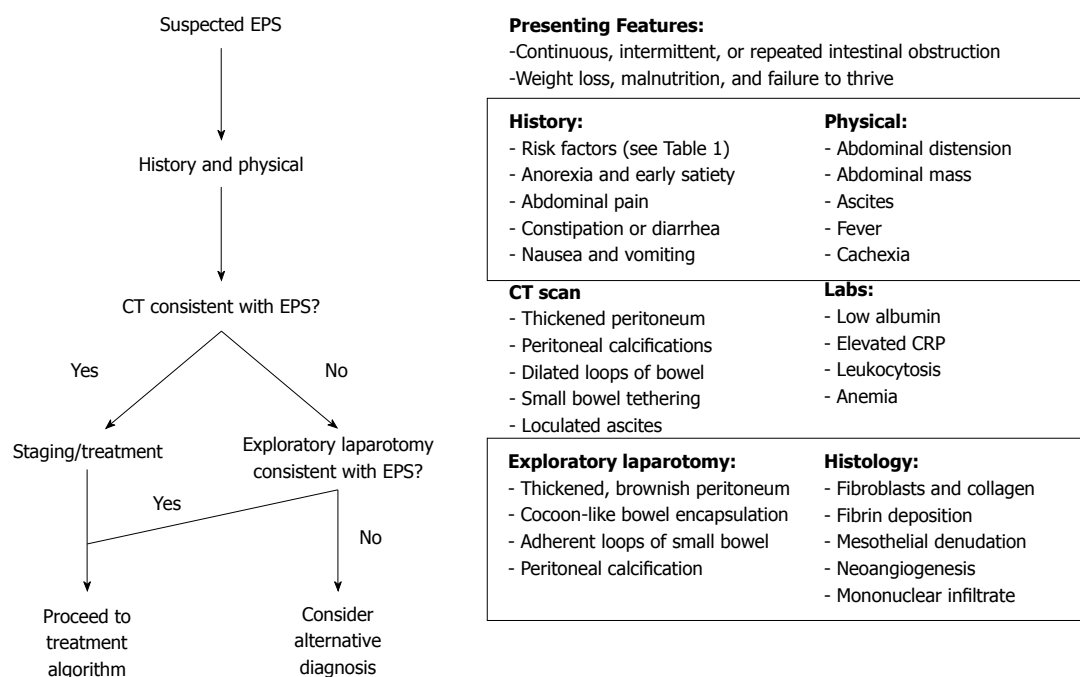
Figure 2 Proposed algorithm for the diagnosis of encapsulating peritoneal sclerosis. The diagnosis of EPS is a clinical one that can be confirmed with imaging or at laparotomy. Various presenting features and symptoms, clues obtained in the history (risk factors) and physical exam can point towards the diagnosis of EPS. CT/MRI findings include thickened peritoneum, calcifications, and may even demonstrate cocooning of the bowel with proximal dilation, as seen in our case. EPS: Encapsulating peritoneal sclerosis

and have a "cauliflower" appearance from compression of tightly adherent loops of bowel by EPS[5]. On ultrasonography, dilated loops of bowel may be matted together and tethered posteriorly or encased by a dense fibrous membrane[5]. Intraperitoneal echogenic strands may cause the bowel wall to appear trilaminar[56,58].

CT scan is currently the best studied and most commonly used imaging technique in the diagnosis of EPS. Small-bowel loops are often tethered together in an enveloping, thickened peritoneum, typically accompanied with proximal bowel dilation[58]. Other radiographic features include loculated ascites, increased density of mesenteric fat, and localized or diffuse peritoneal calcification[56,59]. While the presence of complex loculations could be from intra-abdominal hemorrhage, it should raise suspicion for perforation or sepsis, especially if they contain gas[60]. Increased bowel wall enhancement or thickening indicates ongoing inflammation or transition to transmural fibrosis[58,60]. Magnetic resonance imaging has been used less frequently for diagnosis but likely has similar yields. Advantages include avoidance of ionizing radiation and better delineation of bowel encasement and peritoneal thickening[61].

Laboratory

Laboratory findings in EPS are non-specific and related to underlying infection, malnutrition, and inflammation[30,39,62]. Levels of inflammatory cytokines have been shown to be higher in dialysate in EPS patients compared to PD controls up to years in advance of clinical development of EPS[63-65]. However, no biomarker has been found to be useful in predicting EPS development[64].

Histopathology

Histologic findings in EPS are non-specific and may overlap with findings in simple peritoneal sclerosis or infectious peritonitis[17]. Microscopically, the mesothelial cell layer is denuded with fibroblast proliferation and fibrocollagenous deposition frequently with fibrin deposition[42]. An inflammatory mononuclear cell infiltrate may be present in active inflammation[42]. Podoplanin, a transmembrane glycoprotein found on peritoneal mesothelial cells that binds inflammatory cytokines, helps differentiate EPS from peritoneal sclerosis and peritonitis[45,66]. Generally, a thickened fibrocollagenous membrane in the setting of the previously described clinical syndrome is sufficient for diagnosis.

EPS staging

A staging system has been proposed in the PD-associated EPS literature, based on a combination of clinical, laboratory, and radiographic findings[53,56,57]. Nakamoto *et al*[56] categorized patients with EPS into Stage 1 (pre-EPS), Stage 2 (inflammatory), Stage 3 (encapsulating), and Stage 4 (chronic) based on abdominal symptoms, inflammation, encapsulation, and intestinal findings (Table 2). Different therapeutic approaches have been proposed depending on the stage of disease[53,56,57].

TREATMENT

Treatment of underlying condition

When possible, the underlying condition leading to EPS should be treated. In the case of PD, this involves cessation of peritoneal dialysis and transition to hemodialysis[57]. In non-PD EPS, potential offending medications

Table 2 Stages of peritoneal dialysis-associated encapsulating peritoneal sclerosis with associated clinical, serologic, and radiographic profiles

	Nakamoto 2005		Nakayama 2014	
	Terminology	Clinical findings	Terminology	Clinical findings
Stage 1	Pre-EPS stage	Loss of ultrafiltration capacity	Pre-stage	Abdominal symptoms: Mild
		Development of a high transport		Inflammation: Mild
		Hypoproteinemia		Encapsulation: None
		Bloody dialysate, ascites		
		Calcifications in the peritoneum		
Stage 2	Inflammation stage	Increased CRP, leukocytosis	Inflammatory	Abdominal symptoms: Nausea, diarrhea
		Fever, chills, weight loss, anorexia		Inflammation: Mild to severe
		Diarrhea, ascites		Encapsulation: Partial
Stage 3	Encapsulating stage	Decreased clinical signs of systemic inflammation	Encapsulating	Abdominal symptoms: Periodic ileus
		Early signs of ileus (abdominal pain, nausea, vomiting)		Inflammation: Mild
				Encapsulation: Present
Stage 4	Ileus stage	Anorexia	Chronic	Abdominal symptoms: Persistent ileus
		Complete ileus		Inflammation: None to mild
		Abdominal mass		Encapsulation: Present

should be withdrawn and underlying infection or inflammatory conditions should be treated. Despite withdrawal of the offending agent or treatment of the underlying condition, resolution of EPS is unlikely given its chronic, fibrotic nature and treatment of ongoing inflammation or the underlying fibrosis is frequently necessary[6,8,14,67].

Nutritional support

On diagnosis of EPS, nutritional status should be assessed. While bowel rest and TPN alone are not effective in treating EPS[68], ensuring adequate nutrition is essential. Given obstruction, enteral feeding is often not tolerated and TPN is required[69]. A case series in China, showed preoperative TPN reduced serious postoperative complications and hospital stay[4].

Immunosuppression

The effect of immunosuppression on EPS was first noted in kidney transplant patients who developed improvement in EPS symptoms after institution of immunosuppression[70]. Numerous medications have been tried targeting the inflammatory component of EPS, including corticosteroids, colchicine, azathioprine, cyclosporine, mycophenolate mofetil (MMF), and mammalian target of rapamycin (mTOR) inhibitors[71-76]. Of these, corticosteroids are the best studied and, while the mTOR inhibitors and MMF carry the theoretical advantage of improving fibrosis as well, evidence is anecdotal and largely confined to post-kidney transplant patients with additional indications for immunosuppression[73,75].

Evidence for corticosteroid use is confined to observational studies with inconsistent formulation, dosing, duration, and end points (Table 3). In the earliest study by Kuriyama *et al*[77], all patients who did not receive corticosteroids died within 8 mo, while those who received prednisolone survived at 1-3 years of follow-up with only one requiring surgical intervention. In a larger retrospective case series without a control comparison group, mortality was 25% for stage 4 EPS over the study

period[71] which is lower than what is generally reported in natural history studies[17,50,55].

Despite this, one prospective cohort study found only 38.5% recovered on steroids, while the remainder died or required surgical intervention[68]. Similarly, the largest retrospective study involving steroids found no improvement in median survival, though treatment groups were too heterogeneous for meaningful analysis[55].

While EPS is thought to develop out of an inflammatory insult, active inflammation may not be ongoing at the time of presentation. It is difficult to ascertain which patients are actively inflamed using non-specific markers such as CRP and clinical observation. We recommend targeting immunosuppressive therapy to those thought to have an active inflammatory component after excluding infection. The appropriate dosing and duration of corticosteroids are not established. However the Dutch EPS Registry, in 2011 guidelines, suggests IV methylprednisolone (500-1000 mg/d) for 2-3 d in those who present with acute obstructive symptoms without infection[78]. In those who present with more subacute symptoms without infection, it is reasonable to treat with prednisolone at 0.5-1.0 mg/kg/d for 1 mo and taper over the course of a year[78]. Absence of improvement after 1 mo may be considered a treatment failure and we recommend stopping steroids and considering alternative therapy such as tamoxifen or surgery. In the absence of data, other immunosuppressive medications should be reserved for patients with additional indications such as organ transplantation or strong contraindications to surgery.

Anti-fibrotics

Immunosuppression alone may not be effective in those patients who have already developed signficant fibrosis (Stage 3). Tamoxifen is a selective estrogen receptor modulator (SERM) with strong anti-fibrotic properties related to inhibition of TGF-β, an important cytokine in the fibrosis process[79]. Its successful use in EPS was

Table 3 Summary of studies of interventions in encapsulating peritoneal sclerosis

	Design	Patient population	Treatment	Outcome	Comments
Kuriyama 2001	Retrospective case-control	*n* = 11 Japan PD patients Age - 49.1 yr 27% female	**Steroids** Prednisolone Dose - 0.5 mg/kg/d Duration - NS *n* = 5 **Control** TPN-alone *n* = 6	**Steroids** All remained alive at 1-3 yr after diagnosis. **Control** All died of EPS-related complications within 8 mo of diagnosis.	All control patients were diagnosed prior to 1997 and all who received steroids were diagnosed after 1997.
Kawanishi 2004	Prospective cohort	*n* = 48 Japan PD patients Age - 54.7 yr 25% female	**Steroids** - Prednisolone Dose - 10-40 mg/d Methylprednisolone Dose - 0.5-1.0 g/d Duration – NS *n* = 39 **Surgery** Total enterolysis *n* = 12 **Control** TPN-alone *n* = 3	**Steroids** Recovery - 38.5% Surgery - 15.4% Mortality - 31% **Surgery** Recovery - 58.3 % Mortality - 33% **Control** Recovery - 0% Mortality - 66%	Six steroid patients underwent surgery. Surgical treatment consisted of total enterolysis.
Maruyama 2008	Retrospective case series	*n* = 79 Japan PD patients	**Steroids** Prednisolone Dose – 2.6-60 mg/d Duration - 1-36 mo *n* = 79	**Steroids** Mortality – Stage 2 – 3.6% Stage 3 – 14.3% Stage 4 – 25%	Did not compare to a control group.
Balasubramanian 2009	Retrospective case series	*n* = 111 United Kingdom PD patients Age - 52.0 yr 53% female	**Steroids ± immunosuppression** *n* = 7 **Tamoxifen** *n* = 17 **Tamoxifen + steroids ± immunosuppression** *n* = 8 **Surgery** Adhesiolysis (*n* = 5) jejunostomy (*n* = 1) Small bowel resection (*n* = 1) Ileal-transverse colon bypass (*n* = 1) **Control** No specific drug therapy (*n* = 46)	**Steroids ± immunosuppression** Median survival 7 mo **Tamoxifen** Median survival 15 mo **Tamoxifen + steroids ± immunosuppression** Median survival 14 mo **Surgery** Median survival 17 mo **Control** Median survival 13 mo	Dose and duration of medications not specified. Numerous patients received combinations of therapies. Immunosuppression consisted of azathioprine, cyclosporin, tacrolimus, mycophenolate mofetil, or sirolimus. Unable to analyze statistically due to heterogeneity in groups.
Korte 2011	Retrospective survival analysis	*n* = 63 Netherlands PD patients Age 43.4 yr 50% female	**Tamoxifen** Dose - Dose 10 mg/d to 20 mg twice daily Duration – at least 4 wk *n* = 24 **Control** No tamoxifen *n* =39	**Tamoxifen** Mortality rate - 45.8% **Control** Mortality rate - 74.4%	None underwent surgery in either group. Patients in both groups received steroids, which was not analyzed separately other than noting a trend towards improved mortality in the tamoxifen group.
Kawanishi 2011	Retrospective case series	*n* = 181 Japan PD patients	**Surgery** Total enterolysis *n* = 181	**Surgery** Recurrence – 25.4% Surgical mortality – 7.7% Overall mortality – 35% 0/17 with Noble plication experienced recurrence at 8 mo	Heterogeneous operation types. Those after April 2007 received Noble plication.
Ulmer 2013	Retrospective case series	*n* = 26 Netherlands PD patients Age 54 yr 11% female	**Surgery** Peritonectomy and enterolysis (PEEL)	**Surgery** Major morbidity - 31% Reoperation – 17% Recurrence – 10% Surgical mortality – 10%	8 patients received steroids, 1 tamoxifen, and 1 tacrolimus pre-operatively.

PD: Peritoneal dialysis; EPS: Encapsulating peritoneal sclerosis.

first described by Allaria *et al*[80] in 1999. Similar to corticosteroids, evidence for tamoxifen is limited to observational studies in PD patients (Table 3).

A retrospective Dutch study reported a significant difference in mortality (45.8% *vs* 74.4%, *P* < 0.05) with tamoxifen at 130 mo of follow-up with a trend towards
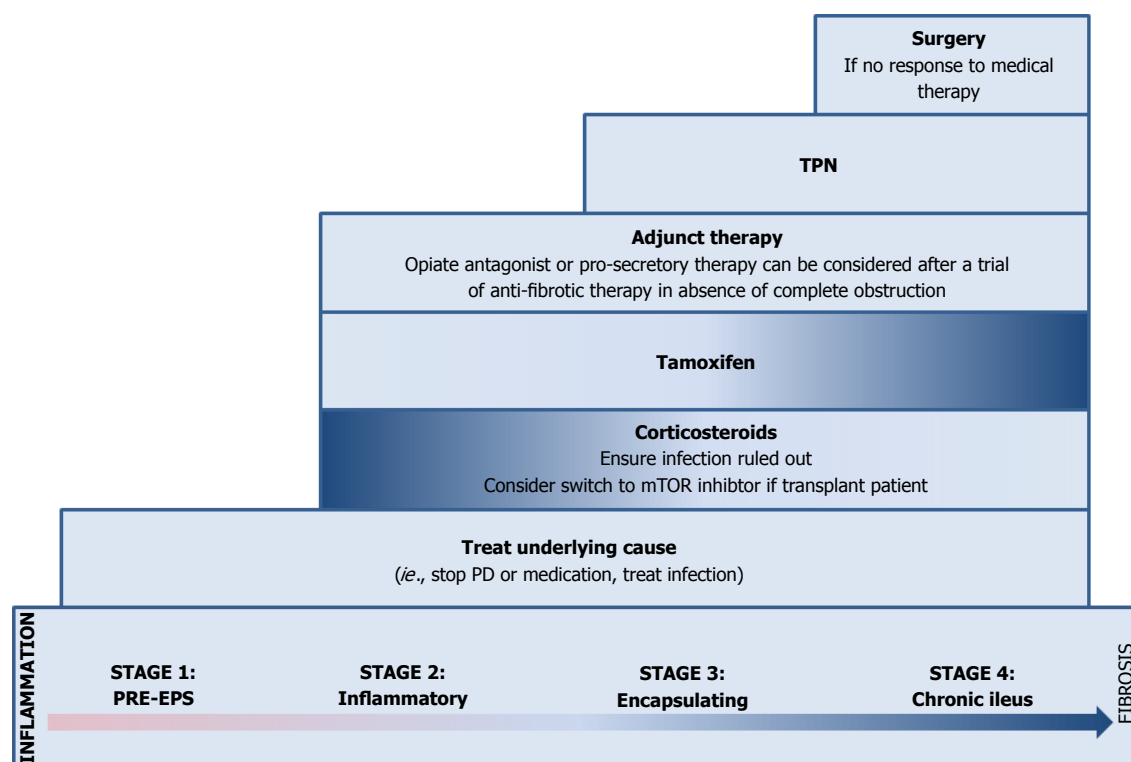
Figure 3 Therapeutic and management approach for encapsulating peritoneal sclerosis. Treatment strategies should be tailored to each patient, depending on the extent and stage of EPS. In all stages, especially during the early stages, the underlying cause should be identified and treated or removed. During the earlier stages (Stage 1-2), the pathophysiology tends to be more inflammatory and the degree of sclerosis tends to be minimal. Thus, after infection has been ruled out, corticosteroids may be of benefit. In the later stages (Stage 3-4), more advanced sclerosis may be present, and patients may start exhibiting signs and symptoms of partial or complete bowel obstruction. In treating abdominal pain, opioids should be avoided. However, this may not always be possible and thus opiate antagonists are recommended in this setting. Tamoxifen plays an increasing rule in the later stages. If poor oral intake or malnutrition is present, total parenteral nutrition may be required. If symptoms are severe and there has been no response to medical therapy, surgical intervention may be considered. EPS: Encapsulating peritoneal sclerosis.

improved survival on survival analysis after adjusting for calendar time, use of corticosteroids, and use of parental nutrition[81]. In contrast, a retrospective study of PD patients from the United Kingdom (UK), did not find any survival benefit with tamoxifen (median 15 mo) compared to no therapy (12 mo) although it was limited by significant treatment heterogeneity[55].

The dose and duration of tamoxifen treatment is not well-defined. Most studies use between 10-40 mg daily. The Dutch EPS Registry suggests starting with 20mg BID[78]. A clinical response is typically seen within 1-6 mo[11,14,82]. Treatment should be continued for at least a year and tapered off thereafter as long as the underlying condition is controlled and the patient has had an adequate clinical and radiologic response. Resolution may be seen in form of clinical improvement and/or evidenced by resolution of peritoneal thickening on follow-up imaging. Potential side effects of tamoxifen including deep venous thrombosis, stroke, hot flashes and endometrial carcinoma should be discussed prior to initiating therapy[83].

Surgical treatment of EPS

Given the time-consuming, hazardous, and technical nature of surgical techniques for EPS, surgery is recommended only in patients who have failed conservative, medical therapy and, if possible, in centers with experi-

ence in such operations. Surgical techniques vary from those with curative intent, such as enterolysis (ablation of fibrotic tissue and lysis of adhesions), to those aimed at addressing a specific complication such as limited lysis of adhesions or resection of perforated or ischemic bowel. The former techniques are preferred. They have lower frequency of symptom recurrence, but are time-consuming, often technically difficult, and carry a risk of bowel injury[84-86].

In a 17-year review of 239 cases in one center in Japan, in which enterolysis alone was primarily employed, mortality was 35.4% (7.7% attributed to post-operative complications and 18.2% to persistent EPS-related complications)[87]. During initial experience, 25.4% required a second operation for persistent obstructive symptoms[85], but after institution of Noble plication (suturing of the intestines to each other) in addition to enterolysis, this improved to 12.3%[88]. More recent experience in other institutions is also encouraging with only 10% requiring reoperation after peritonectomy and enterolysis (PEEL) and 10% mortality at 1 year[86]. Continuation of steroids or tamoxifen post-operatively may reduce recurrence[89].

Dysmotility

While the primary driver of obstructive symptoms in EPS is mechanical and related to adhesions and constriction

within the encapsulating peritoneum, we hypothesize dysmotility may also play a role both through disruption of the myenteric plexus by fibrosis and increased endogenous opioids from activated lymphocytes inhibiting both propulsive motor and secretory activity in the gut[66,90,91]. Successful use of methylnaltrexone to combat inflammation-associated dysmotility has been described in anti-Hu-associated intestinal pseudo-obstruction[92]. We suggest use of a μ-opioid antagonist and a guanylate cyclase C agonist in patients who remain symptomatic, but have already been started on appropriate anti-fibrotic therapy (Figure 3).

CONCLUSION

Encapsulating peritoneal sclerosis is a rare, but devastating condition associated with high morbidity and mortality. A high index of suspicion is warranted in patients with unexplained recurrent symptoms of bowel obstruction. A careful history should be obtained to identify patients with known risk factors. EPS can be divided into primary (idiopathic) or secondary. Implicated triggers may include medications, infections, chronic mechanical or chemical irritation, especially in patients on peritoneal dialysis, or in various other chronic, inflammatory diseases (cirrhosis, history of organ transplant, gynecologic or rheumatologic conditions). Diagnosis is clinical, and can be confirmed by x-ray or laparotomy. Treatment should be directed at the underlying condition, optimizing nutrition, and corticosteroids or tamoxifen alone-or in combination-depending on disease state and contraindications. In patients who have failed conservative medical therapy, surgical enterolysis should be considered.

Prior studies have been limited by inconsistent definitions and staging of EPS, and all treatment studies have been observational with variable dosage, duration of treatment, and outcomes. Since treatment may vary based on disease stage, future studies should appropriately document disease stage when assessing treatment. Long-term longitudinal data from past case series may shed more light on the natural history of EPS and mortality benefits of certain treatments. A gap in knowledge still exists in our understanding of the underlying pathogenesis of EPS and fibrosis, and will need to be bridged in order to develop effective therapies.

REFERENCES

1   **Kawaguchi Y**, Kawanishi H, Mujais S, Topley N, Oreopoulos DG. Encapsulating peritoneal sclerosis: definition, etiology, diagnosis, and treatment. International Society for Peritoneal Dialysis Ad Hoc Committee on Ultrafiltration Management in Peritoneal Dialysis. *Perit Dial Int* 2000; **20** Suppl 4: S43-S55 [PMID: 11098928]
2   **Owtschinnikow PJ**. Peritonitis chronica fibrosa incapsulata. *Arch für Klin Chir* 1907; **83**: 623-634
3   **Casas JD**, Mariscal A, Martínez N. Peritoneal encapsulation: CT appearance. *AJR Am J Roentgenol* 1998; **171**: 1017-1019 [PMID: 9762988 DOI: 10.2214/ajr.171.4.9762988]
4   **Li N**, Zhu W, Li Y, Gong J, Gu L, Li M, Cao L, Li J. Surgical treatment and perioperative management of idiopathic abdominal cocoon: single-center review of 65 cases. *World J Surg* 2014; **38**: 1860-1867 [PMID: 24519587 DOI: 10.1007/s00268-014-2458-6]
5   **Akbulut S**. Accurate definition and management of idiopathic sclerosing encapsulating peritonitis. *World J Gastroenterol* 2015; **21**: 675-687 [PMID: 25593498 DOI: 10.3748/wjg.v21.i2.675]
6   **Eltringham WK**, Espiner HJ, Windsor CW, Griffiths DA, Davies JD, Baddeley H, Read AE, Blunt RJ. Sclerosing peritonitis due to practolol: a report on 9 cases and their surgical management. *Br J Surg* 1977; **64**: 229-235 [PMID: 856375 DOI: 10.1002/bjs.1800640402]
7   **Sarker S**, Kodali S, Weber F. A new meaning to butterflies in the stomach. *Gastroenterology* 2015; **148**: e12-e13 [PMID: 25450077 DOI: 10.1053/j.gastro.2014.07.037]
8   **Sachdev A**, Usatoff V, Thaow C. Sclerosing encapsulating peritonitis and methotrexate. *Aust N Z J Obstet Gynaecol* 2006; **46**: 58-59 [PMID: 16441697 DOI: 10.1111/j.1479-828X.2006.00517.x]
9   **Nauen DW**, Martin A, Katz A, Cohen D, Ranganathan S. A case of luteinizing thecoma with sclerosing peritonitis: revisiting a link with anti-epileptic drugs. *Pediatr Blood Cancer* 2010; **54**: 470-472 [PMID: 19847882 DOI: 10.1002/pbc.22325]
10 **Sharma V**, Mandavdhare HS, Rana SS, Singh H, Kumar A, Gupta R. Role of conservative management in tubercular abdominal cocoon: a case series. *Infection* 2017; **45**: 601-606 [PMID: 28341896 DOI: 10.1007/s15010-017-1012-5]
11 **Simbli MA**, Niaz FA, Al-Wakeel JS. Encapsulating peritoneal sclerosis in a peritoneal dialysis patient presenting with complicated Mycobacterium fortuitum peritonitis. *Saudi J Kidney Dis Transpl* 2012; **23**: 635-641 [PMID: 22569461]
12 **Hsu YH**, Hsia CC, Tsai DM, Tu HY, Hung KY, Huang JW. Development of encapsulating peritoneal sclerosis following bacterial peritonitis in a peritoneal dialysis patient. *Am J Kidney Dis* 2010; **55**: 198-202 [PMID: 19782452 DOI: 10.1053/j.ajkd.2009.06.043]
13 **Browne LP**, Patel J, Guillerman RP, Hanson IC, Cass DL. Abdominal cocoon: a unique presentation in an immunodeficient infant. *Pediatr Radiol* 2012; **42**: 263-266 [PMID: 21713442 DOI: 10.1007/s00247-011-2135-y]
14 **Gupta S**, Woodrow G. Successful treatment of fulminant encapsulating peritoneal sclerosis following fungal peritonitis with tamoxifen. *Clin Nephrol* 2007; **68**: 125-129 [PMID: 17722714 DOI: 10.5414/CNP68125]
15 **Tan J**, Manickam R, Pisharam J, Telisinghe P, Chong VH. Mucormycosis--a possible trigger pathogen for encapsulating peritoneal sclerosis. *Perit Dial Int* 2012; **32**: 479-481 [PMID: 22859845 DOI: 10.3747/pdi.2011.00241]
16 **Salamone G**, Atzeni J, Agrusa A, Gulotta G. A rare case of abdominal cocoon. *Ann Ital Chir* 2013; **84** [PMID: 24141102]
17 **Brown EA**, Bargman J, van Biesen W, Chang MY, Finkelstein FO, Hurst H, Johnson DW, Kawanishi H, Lambie M, de Moraes TP, Morelle J, Woodrow G. Length of Time on Peritoneal Dialysis and Encapsulating Peritoneal Sclerosis - Position Paper for ISPD: 2017 Update. *Perit Dial Int* 2017; **37**: 362-374 [PMID: 28676507 DOI: 10.3747/pdi.2017.00018]
18 **Takebayashi K**, Sonoda H, Shimizu T, Ohta H, Ishida M, Mekata E, Endo Y, Tani T, Tani M. Successful surgical approach for a patient with encapsulating peritoneal sclerosis after hyperthermic intraperitoneal chemotherapy: a case report and literature review. *BMC Surg* 2014; **14**: 57 [PMID: 25160862 DOI: 10.1186/1471-2482-14-57]
19 **Wirnsberger GH**, Ganser K, Domej W, Sauseng G, Moore D, Moczygemba M, Krejs GJ. Sclerosing encapsulating peritonitis: differential diagnosis to peritoneal encapsulation and abdominal cocoon--a case report. *Z Gastroenterol* 1992; **30**: 534-537 [PMID: 1413936]
20 **Sigaroudinia MO**, Baillie C, Ahmed S, Mallucci C. Sclerosing encapsulating peritonitis--a rare complication of ventriculoperitoneal shunts. *J Pediatr Surg* 2008; **43**: E31-E33 [PMID: 18485933 DOI: 10.1016/j.jpedsurg.2008.01.019]
21 **Lin CH**, Yu JC, Chen TW, Chan DC, Chen CJ, Hsieh CB. Sclerosing encapsulating peritonitis in a liver transplant patient: a case report. *World J Gastroenterol* 2005; **11**: 5412-5413 [PMID: 16149160 DOI:

10.3748/wjg.v11.i34.5412]

22 **Keating JP**, Neill M, Hill GL. Sclerosing encapsulating peritonitis after intraperitoneal use of povidone iodine. *Aust N Z J Surg* 1997; **67**: 742-744 [PMID: 9322732 DOI: 10.1111/j.1445-2197.1997.tb07126.x]

23 **Kaur S**, Doley RP, Chabbhra M, Kapoor R, Wig J. Post trauma abdominal cocoon. *Int J Surg Case Rep* 2015; **7C**: 64-65 [PMID: 25590647 DOI: 10.1016/j.ijscr.2014.10.081]

24 **Kaman L**, Iqbal J, Thenozhi S. Sclerosing encapsulating peritonitis: complication of laparoscopic cholecystectomy. *J Laparoendosc Adv Surg Tech A* 2010; **20**: 253-255 [PMID: 20374014 DOI: 10.1089/lap.2010.0024]

25 **Árnadóttir M**, Jónasson JG, Indridason ÓS. Encapsulating peritoneal sclerosis following a peritoneal foreign body reaction to Dacron fibres-a case report. *NDT Plus* 2011; **4**: 107-109 [PMID: 25984126 DOI: 10.1093/ndtplus/sfq202]

26 **Andrion A**, Pira E, Mollo F. Peritoneal plaques and asbestos exposure. *Arch Pathol Lab Med* 1983; **107**: 609-610 [PMID: 6688720]

27 **Castelli MJ**, Armin AR, Husain A, Orfei E. Fibrosing peritonitis in a drug abuser. *Arch Pathol Lab Med* 1985; **109**: 767-769 [PMID: 3839380]

28 **Wakabayashi H**, Okano K, Suzuki Y. Clinical challenges and images in GI. Image 2. Perforative peritonitis on sclerosing encapsulating peritonitis (abdominal cocoon) in a patient with alcoholic liver cirrhosis. *Gastroenterology* 2007; **132**: 854, 1210 [PMID: 17383416 DOI: 10.1053/j.gastro.2007.01.060]

29 **Lee KW**, Cho CW, Lee N, Lee S, Kim JM, Choi GS, Kwon CH, Joh JW, Lee SK. Encapsulating peritoneal sclerosis in liver transplant recipients: a report of 2 cases. *Ann Surg Treat Res* 2017; **92**: 164-167 [PMID: 28289671 DOI: 10.4174/astr.2017.92.3.164]

30 **Rumbo C**, Zambernardi A, Cabanne A, Rumbo M, Gondolesi G. Sclerosing peritonitis, a rare complication after intestinal transplant. Report of one case successfully treated with adjustment of immunosuppression. *Pediatr Transplant* 2013; **17**: E125-E129 [PMID: 23902605 DOI: 10.1111/petr.12110]

31 **Morrow EH**, Gallo AE, Melcher ML. Sclerosing peritonitis after kidney transplantation: a not-so-silky cocoon. *Dig Dis Sci* 2011; **56**: 307-310 [PMID: 21063775 DOI: 10.1007/s10620-010-1471-3]

32 **Frigerio L**, Taccagni GL, Mariani A, Mangili G, Ferrari A. Idiopathic sclerosing peritonitis associated with florid mesothelial hyperplasia, ovarian fibromatosis, and endometriosis: a new disorder of abdominal mass. *Am J Obstet Gynecol* 1997; **176**: 721-722 [PMID: 9077640 DOI: 10.1016/S0002-9378(97)70581-7]

33 **Bahar B**, Hu Z, Szpaderska A, Liotta M, Potkul RK, Smith D, Erşahin Ç. Fatal case of luteinized thecoma with sclerosing peritonitis in a 40-year-old woman. *Int J Gynecol Pathol* 2014; **33**: 30-34 [PMID: 24300532 DOI: 10.1097/PGP.0b013e31827d1a65]

34 **Walker J**, Moss EL, Ganesan R, Hirschowitz L. Sclerosing peritonitis associated with a luteinized adult granulosa cell tumor. *Int J Gynecol Pathol* 2012; **31**: 141-144 [PMID: 22317869 DOI: 10.1097/PGP.0b013e3182307b28]

35 **Fossey SJ**, Simson JN. Sclerosing encapsulating peritonitis secondary to dermoid cyst rupture: a case report. *Ann R Coll Surg Engl* 2011; **93**: e39-e40 [PMID: 21943446 DOI: 10.1308/147870811X582495]

36 **Ngô Y**, Messing B, Marteau P, Nouël O, Pasquiou A, Lavergne A, Rambaud JC. Peritoneal sarcoidosis. An unrecognized cause of sclerosing peritonitis. *Dig Dis Sci* 1992; **37**: 1776-1780 [PMID: 1425080 DOI: 10.1007/BF01299875]

37 **Pepels MJ**, Peters FP, Mebis JJ, Ceelen TL, Hoofwijk AG, Erdkamp FL. Sclerosing peritonitis: an unusual cause of ascites in a patient with systemic lupus erythematosus. *Neth J Med* 2006; **64**: 346-349 [PMID: 17057274]

38 **Dabak R**, Uygur-Bayramiçli O, Aydin DK, Dolapçioglu C, Gemici C, Erginel T, Turan C, Karadayi N. Encapsulating peritonitis and familial Mediterranean fever. *World J Gastroenterol* 2005; **11**: 2844-2846 [PMID: 15884137 DOI: 10.3748/wjg.v11.i18.2844]

39 **Alston H**, Fan S, Nakayama M. Encapsulating Peritoneal Sclerosis. *Semin Nephrol* 2017; **37**: 93-102 [PMID: 28153198 DOI: 10.1016/j.semnephrol.2016.10.010]

40 **Johnson DW**, Cho Y, Livingston BE, Hawley CM, McDonald SP, Brown FG, Rosman JB, Bannister KM, Wiggins KJ. Encapsulating peritoneal sclerosis: incidence, predictors, and outcomes. *Kidney Int* 2010; **77**: 904-912 [PMID: 20375981 DOI: 10.1038/ki.2010.16]

41 **Kawanishi H**, Moriishi M. Epidemiology of encapsulating peritoneal sclerosis in Japan. *Perit Dial Int* 2005; **25** Suppl 4: S14-S18 [PMID: 16300268]

42 **Honda K**, Oda H. Pathology of encapsulating peritoneal sclerosis. *Perit Dial Int* 2005; **25** Suppl 4: S19-S29 [PMID: 16300269]

43 **Abrahams AC**, Habib SM, Dendooven A, Riser BL, van der Veer JW, Toorop RJ, Betjes MG, Verhaar MC, Watson CJ, Nguyen TQ, Boer WH. Patients with encapsulating peritoneal sclerosis have increased peritoneal expression of connective tissue growth factor (CCN2), transforming growth factor-β1, and vascular endothelial growth factor. *PLoS One* 2014; **9**: e112050 [PMID: 25384022 DOI: 10.1371/journal.pone.0112050]

44 **Lambie MR**, Chess J, Summers AM, Williams PF, Topley N, Davies SJ; GLOBAL Fluid Study Investigators. Peritoneal inflammation precedes encapsulating peritoneal sclerosis: results from the GLOBAL Fluid Study. *Nephrol Dial Transplant* 2016; **31**: 480-486 [PMID: 26908833 DOI: 10.1093/ndt/gfv440]

45 **Braun N**, Alscher DM, Fritz P, Edenhofer I, Kimmel M, Gaspert A, Reimold F, Bode-Lesniewska B, Ziegler U, Biegger D, Wüthrich RP, Segerer S. Podoplanin-positive cells are a hallmark of encapsulating peritoneal sclerosis. *Nephrol Dial Transplant* 2011; **26**: 1033-1041 [PMID: 20709739 DOI: 10.1093/ndt/gfq488]

46 **Lopez-Anton M**, Lambie M, Lopez-Cabrera M, Schmitt CP, Ruiz-Carpio V, Bartosova M, Schaefer B, Davies S, Stone T, Jenkins R, Taylor PR, Topley N, Bowen T, Fraser D. miR-21 Promotes Fibrogenesis in Peritoneal Dialysis. *Am J Pathol* 2017; **187**: 1537-1550 [PMID: 28495592 DOI: 10.1016/j.ajpath.2017.03.007]

47 **Reimold FR**, Braun N, Zsengellér ZK, Stillman IE, Karumanchi SA, Toka HR, Latus J, Fritz P, Biegger D, Segerer S, Alscher MD, Bhasin MK, Alper SL. Transcriptional patterns in peritoneal tissue of encapsulating peritoneal sclerosis, a complication of chronic peritoneal dialysis. *PLoS One* 2013; **8**: e56389 [PMID: 23418565 DOI: 10.1371/journal.pone.0056389]

48 **Maruyama Y**, Numata M, Nakayama M, Matsuo N, Nordfors L, Hosoya T, Lindholm B. Relationship between the -374T/A receptor of advanced glycation end products gene polymorphism and peritoneal solute transport status at the initiation of peritoneal dialysis. *Ther Apher Dial* 2007; **11**: 301-305 [PMID: 17661837 DOI: 10.1111/j.1744-9987.2007.00473.x]

49 **Clement PB**, Young RH, Hanna W, Scully RE. Sclerosing peritonitis associated with luteinized thecomas of the ovary. A clinicopathological analysis of six cases. *Am J Surg Pathol* 1994; **18**: 1-13 [PMID: 8279621 DOI: 10.1097/00000478-199401000-00001]

50 **Betjes MG**, Habib SM, Boeschoten EW, Hemke AC, Struijk DG, Westerhuis R, Abrahams AC, Korte MR. Significant Decreasing Incidence of Encapsulating Peritoneal Sclerosis in the Dutch Population of Peritoneal Dialysis Patients. *Perit Dial Int* 2017; **37**: 230-234 [PMID: 28360369 DOI: 10.3747/pdi.2016.00109]

51 **Hoshii S**, Honda M, Itami N, Oh S, Matsumura C, Moriya S, Mori M, Hatae K, Ito Y, Karashima S. Sclerosing encapsulating peritonitis in pediatric peritoneal dialysis patients. *Pediatr Nephrol* 2000; **14**: 275-279 [PMID: 10775068 DOI: 10.1007/s004670050758]

52 **Phelan PJ**, Walshe JJ, Al-Aradi A, Garvey JP, Finnegan K, O'Kelly P, McWilliams J, Ti JP, Morrin MM, Morgan N, Conlon PJ. Encapsulating peritoneal sclerosis: experience of a tertiary referral center. *Ren Fail* 2010; **32**: 459-463 [PMID: 20446784 DOI: 10.3109/08860221003658274]

53 **Nakayama M**, Miyazaki M, Honda K, Kasai K, Tomo T, Nakamoto H, Kawanishi H. Encapsulating peritoneal sclerosis in the era of a multi-disciplinary approach based on biocompatible solutions: the NEXT-PD study. *Perit Dial Int* 2014; **34**: 766-774 [PMID: 24497585 DOI: 10.3747/pdi.2013.00074]

54 **Rigby RJ**, Hawley CM. Sclerosing peritonitis: the experience in Australia. *Nephrol Dial Transplant* 1998; **13**: 154-159 [PMID: 9481732 DOI: 10.1093/ndt/13.1.154]

55 **Balasubramaniam G**, Brown EA, Davenport A, Cairns H, Cooper

B, Fan SL, Farrington K, Gallagher H, Harnett P, Krausze S, Steddon S. The Pan-Thames EPS study: treatment and outcomes of encapsulating peritoneal sclerosis. *Nephrol Dial Transplant* 2009; **24**: 3209-3215 [PMID: 19211652 DOI: 10.1093/ndt/gfp008]

56 **Nakamoto H**. Encapsulating peritoneal sclerosis -- a clinician's approach to diagnosis and medical treatment. *Perit Dial Int* 2005; **25** Suppl 4: S30-S38 [PMID: 16300270]

57 **Moinuddin Z**, Summers A, Van Dellen D, Augustine T, Herrick SE. Encapsulating peritoneal sclerosis-a rare but devastating peritoneal disease. *Front Physiol* 2015; **5**: 470 [PMID: 25601836 DOI: 10.3389/fphys.2014.00470]

58 **Ti JP**, Al-Aradi A, Conlon PJ, Lee MJ, Morrin MM. Imaging features of encapsulating peritoneal sclerosis in continuous ambulatory peritoneal dialysis patients. *AJR Am J Roentgenol* 2010; **195**: W50-W54 [PMID: 20566781 DOI: 10.2214/AJR.09.3175]

59 **Stuart S**, Stott D, Goode A, Cash CJ, Davenport A. Can radiological assessment of abdominal computerized scans diagnose encapsulating peritoneal sclerosis in long-term peritoneal dialysis patients? *Nephrology* (Carlton) 2017; **22**: 19-24 [PMID: 26730546 DOI: 10.1111/nep.12718]

60 **Upponi S**, Butler AJ, Watson CJ, Shaw AS. Encapsulating peritoneal sclerosis--correlation of radiological findings at CT with underlying pathogenesis. *Clin Radiol* 2014; **69**: 103-109 [PMID: 24209872 DOI: 10.1016/j.crad.2013.09.004]

61 **Jovani M**, Baticci F, Bonifacio C, Omodei PD, Malesci A. Abdominal cocoon or idiopathic encapsulating peritoneal sclerosis: magnetic resonance imaging. *Dig Liver Dis* 2014; **46**: 192-193 [PMID: 24055233 DOI: 10.1016/j.dld.2013.08.136]

62 **Cho R**, Ghag D, Karim MA, Lo C. Encapsulating peritoneal sclerosis: surgery, sustained drug therapy and treatment of recurrence at 1 year. *BMJ Case Rep* 2015; **2015**: [PMID: 26561223 DOI: 10.1136/bcr-2015-211490]

63 **Lopes Barreto D**, Struijk DG, Krediet RT. Peritoneal effluent MMP-2 and PAI-1 in encapsulating peritoneal sclerosis. *Am J Kidney Dis* 2015; **65**: 748-753 [PMID: 25530106 DOI: 10.1053/j.ajkd.2014.10.022]

64 **Goodlad C**, Tam FW, Ahmad S, Bhangal G, North BV, Brown EA. Dialysate cytokine levels do not predict encapsulating peritoneal sclerosis. *Perit Dial Int* 2014; **34**: 594-604 [PMID: 24584593 DOI: 10.3747/pdi.2012.00305]

65 **Ahmad S**, North BV, Qureshi A, Malik A, Bhangal G, Tarzi RM, Brown EA, Tam FW. CCL18 in peritoneal dialysis patients and encapsulating peritoneal sclerosis. *Eur J Clin Invest* 2010; **40**: 1067-1073 [PMID: 20695883 DOI: 10.1111/j.1365-2362.2010.02353.x]

66 **Braun N**, Fritz P, Ulmer C, Latus J, Kimmel M, Biegger D, Ott G, Reimold F, Thon KP, Dippon J, Segerer S, Alscher MD. Histological criteria for encapsulating peritoneal sclerosis - a standardized approach. *PLoS One* 2012; **7**: e48647 [PMID: 23144917 DOI: 10.1371/journal.pone.0048647]

67 **Lalloo S**, Krishna D, Maharajh J. Case report: abdominal cocoon associated with tuberculous pelvic inflammatory disease. *Br J Radiol* 2002; **75**: 174-176 [PMID: 11893642 DOI: 10.1259/bjr.75.890.750174]

68 **Kawanishi H**, Kawaguchi Y, Fukui H, Hara S, Imada A, Kubo H, Kin M, Nakamoto M, Ohira S, Shoji T. Encapsulating peritoneal sclerosis in Japan: a prospective, controlled, multicenter study. *Am J Kidney Dis* 2004; **44**: 729-737 [PMID: 15384025 DOI: 10.1053/j.ajkd.2004.06.020]

69 **de Freitas D**, Jordaan A, Williams R, Alderdice J, Curwell J, Hurst H, Hutchison A, Brenchley PE, Augustine T, Summers AM. Nutritional management of patients undergoing surgery following diagnosis with encapsulating peritoneal sclerosis. *Perit Dial Int* 2008; **28**: 271-276 [PMID: 18474920]

70 **Junor BJ**, McMillan MA. Immunosuppression in sclerosing peritonitis. *Adv Perit Dial* 1993; **9**: 187-189 [PMID: 8105920]

71 **Maruyama Y**, Nakayama M. Encapsulating peritoneal sclerosis in Japan. *Perit Dial Int* 2008; **28** Suppl 3: S201-S204 [PMID: 18552256]

72 **Ceri M**, Unverdi S, Dogan M, Unverdi H, Karaca G, Kocak G,
Kurultak I, Akbal E, Can M, Duranay M. Effect of sirolimus on the regression of peritoneal sclerosis in an experimental rat model. *Int Urol Nephrol* 2012; **44**: 977-982 [PMID: 22528580 DOI: 10.1007/s11255-012-0167-3]

73 **Sud R**, Garry L, Spicer ST, Allen RD, Eris JM, Wyburn K, Verran D, Cooper CL, Chadban S. A role for everolimus in post-transplant encapsulating peritoneal sclerosis: first case report. *Nephrology (Carlton)* 2014; **19** Suppl 1: 27-30 [PMID: 24460661 DOI: 10.1111/nep.12196]

74 **Romagnoli J**, Pedroso JA, Salerno MP, Favi E, Spagnoletti G, Citterio F. Posttransplant encapsulating peritoneal sclerosis, long-term success with everolimus and low-dose CNI: a case report. *Transplant Proc* 2014; **46**: 2368-2370 [PMID: 25242790 DOI: 10.1016/j.transproceed.2014.07.060]

75 **Huddam B**, Başaran M, Koçak G, Azak A, Yalçın F, Reyhan NH, Duranay M. The use of mycophenolate mofetil in experimental encapsulating peritoneal sclerosis. *Int Urol Nephrol* 2015; **47**: 1423-1428 [PMID: 26159779 DOI: 10.1007/s11255-015-1015-z]

76 **Bozkurt D**, Bicak S, Sipahi S, Taskin H, Hur E, Ertilav M, Sen S, Duman S. The effects of colchicine on the progression and regression of encapsulating peritoneal sclerosis. *Perit Dial Int* 2008; **28** Suppl 5: S53-S57 [PMID: 19008543]

77 **Kuriyama S**, Tomonari H. Corticosteroid therapy in encapsulating peritoneal sclerosis. *Nephrol Dial Transplant* 2001; **16**: 1304-1305 [PMID: 11390748 DOI: 10.1093/ndt/16.6.1304]

78 **Habib SM**, Betjes MG, Fieren MW, Boeschoten EW, Abrahams AC, Boer WH, Struijk DG, Ruger W, Krikke C, Westerhuis R, de Sévaux RG, van der Sande FM, Gaasbeek A, Korte MR; Eps Registry. Management of encapsulating peritoneal sclerosis: a guideline on optimal and uniform treatment. *Neth J Med* 2011; **69**: 500-507 [PMID: 22173363]

79 **Loureiro J**, Aguilera A, Selgas R, Sandoval P, Albar-Vizcaíno P, Pérez-Lozano ML, Ruiz-Carpio V, Majano PL, Lamas S, Rodríguez-Pascual F, Borras-Cuesta F, Dotor J, López-Cabrera M. Blocking TGF-β1 protects the peritoneal membrane from dialysate-induced damage. *J Am Soc Nephrol* 2011; **22**: 1682-1695 [PMID: 21742730 DOI: 10.1681/ASN.2010111197]

80 **Allaria PM**, Giangrande A, Gandini E, Pisoni IB. Continuous ambulatory peritoneal dialysis and sclerosing encapsulating peritonitis: tamoxifen as a new therapeutic agent? *J Nephrol* 1999; **12**: 395-397 [PMID: 10626830]

81 **Korte MR**, Fieren MW, Sampimon DE, Lingsma HF, Weimar W, Betjes MG; investigators of the Dutch Multicentre EPS Study. Tamoxifen is associated with lower mortality of encapsulating peritoneal sclerosis: results of the Dutch Multicentre EPS Study. *Nephrol Dial Transplant* 2011; **26**: 691-697 [PMID: 20584735 DOI: 10.1093/ndt/gfq362]

82 **Moustafellos P**, Hadjianastassiou V, Roy D, Velzeboer NE, Maniakyn N, Vaidya A, Friend PJ. Tamoxifen therapy in encapsulating sclerosing peritonitis in patients after kidney transplantation. *Transplant Proc* 2006; **38**: 2913-2914 [PMID: 17112862 DOI: 10.1016/j.transproceed.2006.08.179]

83 **Fisher B**, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, Bevers TB, Kavanah MT, Atkins JN, Margolese RG, Runowicz CD, James JM, Ford LG, Wolmark N. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J Natl Cancer Inst* 2005; **97**: 1652-1662 [PMID: 16288118 DOI: 10.1093/jnci/dji372]

84 **Célicout B**, Levard H, Hay J, Msika S, Fingerhut A, Pelissier E. Sclerosing encapsulating peritonitis: early and late results of surgical management in 32 cases. French Associations for Surgical Research. *Dig Surg* 1998; **15**: 697-702 [PMID: 9845640 DOI: 10.1159/000018681]

85 **Kawanishi H**, Ide K, Yamashita M, Shimomura M, Moriishi M, Tsuchiya S, Dohi K. Surgical techniques for prevention of recurrence after total enterolysis in encapsulating peritoneal sclerosis. *Adv Perit Dial* 2008; **24**: 51-55 [PMID: 18986001]

86 **Ulmer C**, Braun N, Rieber F, Latus J, Hirschburger S, Emmel J, Alscher MD, Steurer W, Thon KP. Efficacy and morbidity of surgical therapy in late-stage encapsulating peritoneal sclerosis. *Surgery* 2013;

153: 219-224 [PMID: 22981361 DOI: 10.1016/j.surg.2012.07.033]

87   **Kawanishi H**, Shintaku S, Moriishi M, Dohi K, Tsuchiya S. Seventeen years' experience of surgical options for encapsulating peritoneal sclerosis. *Adv Perit Dial* 2011; **27**: 53-58 [PMID: 22073830]

88   **Kawanishi H**. Surgical and medical treatments of encapsulation peritoneal sclerosis. *Contrib Nephrol* 2012; **177**: 38-47 [PMID: 22613913 DOI: 10.1159/000336934]

89   **Lo WK**, Kawanishi H. Encapsulating peritoneal sclerosis--medical and surgical treatment. *Perit Dial Int* 2009; **29** Suppl 2: S211-S214 [PMID: 19270221]

90   **Kurz A**, Sessler DI. Opioid-induced bowel dysfunction: pathophysiology and potential new therapies. *Drugs* 2003; **63**: 649-671 [PMID: 12656645 DOI: 10.2165/00003495-200363070-000 03]

91   **Ketwaroo GA**, Cheng V, Lembo A. Opioid-induced bowel dysfunction. *Curr Gastroenterol Rep* 2013; **15**: 344 [PMID: 23900996 DOI: 10.1007/s11894-013-0344-2]

92   **Zhang C**, Patel NJ, Jacobs WC, Ullman S, Berzin TM, Chuttani R, Lembo AJ, Wolf JL. Successful Treatment with Methylnaltrexone and IVIG for Paraneoplastic Syndrome-Associated Intestinal Pseudo-Obstruction. *Gastroenterol Hepatol* (NY) 2013; **9**: 48-51 [PMID: 24707235]

MINIREVIEWS

Considerations for bariatric surgery in patients with cirrhosis

George Boon-Bee Goh, Philip R Schauer, Arthur J McCullough

George Boon-Bee Goh, Department of Gastroenterology and Hepatology, Singapore General Hospital, Singapore 169608, Singapore

George Boon-Bee Goh, Duke-NUS Graduate Medical School, Singapore 169608, Singapore

Philip R Schauer, Bariatric and Metabolic Institute, Cleveland Clinic, Cleveland, OH 44195, United States

Arthur J McCullough, Department of Gastroenterology, Cleveland Clinic, Cleveland, OH 44195, United States

Arthur J McCullough, Department of Pathobiology, Cleveland Clinic, Cleveland, OH 44195, United States

ORCID number: George Boon-Bee Goh (0000-0001-8221-5299); Philip R Schauer (0000-0001-6258-5604); Arthur J McCullough (0000-0002-4906-0034).

Author contributions: All the authors contributed to the writing of this review.

Conflict-of-interest statement: All Authors declare no conflict of interest for this manuscript.

Correspondence to: George Boon-Bee Goh, MBBS, MRCP, Adjunct Professor, Department of Gastroenterology and Hepatology, Singapore General Hospital, 20 College Road, Academia, Singapore 169856, Singapore. goh.boon.bee@singhealth.com.sg
Telephone: +65-63266137

Abstract

With the ever increasing global obesity pandemic, clinical burden from obesity related complications are anticipated in parallel. Bariatric surgery, a treatment approved for weight loss in morbidly obese patients, has reported to be associated with good outcomes, such as reversal of type two diabetes mellitus and reducing all-cause mortality on a long term basis. However, complications from bariatric surgery have similarly been reported. In particular, with the onslaught of non-alcoholic fatty liver disease (NAFLD) epidemic, in associated with obesity and metabolic syndrome, there is increasing prevalence of NAFLD related liver cirrhosis, which potentially connotes more risk of specific complications for surgery. Bariatric surgeons may encounter, either expectedly or unexpectedly, patients with non-alcoholic steatohepatitis (NASH) and NASH related cirrhosis more frequently. As such, the issues and considerations surrounding their medical care/surgery warrant careful deliberation to ensure the best outcomes. These considerations include severity of cirrhosis, liver synthetic function, portal hypertension and the impact of surgical factors. This review explores these considerations comprehensively and emphasizes the best approach to managing cirrhotic patients in the context of bariatric surgery.

Key words: Cirrhosis; Portal hypertension; Non-alcoholic fatty liver disease; Bariatric surgery; Complications

Core tip: Bariatric surgery can be performed in patients with well compensated cirrhosis, typically of Child's A status, with minimal complication risks from surgical or hepatic factors. Patients need to be carefully selected and optimised, while surgical technique and modality also play equally important roles. With the onslaught of the non-alcoholic fatty liver disease epidemic and anticipated increase in patients with non-alcoholic steatohepatitis cirrhosis, bariatric surgery may provide an elixir as part of the armamentarium of therapeutic options.

INTRODUCTION

The global obesity pandemic continues unabated, accompanied with increasing healthcare and economic burden[1]. In 2005, 23.2% of the world's adult population were overweight and 9.8% were considered obese, translating to 937 million and 396 million overweight and obese adults, respectively. Projections have estimated the prevalence of overweight and obese adults to increase to 1.35 billion and 573 million adults by 2030 respectively[2]. Obesity is commonly associated with a plethora of chronic diseases, among them being non-alcoholic fatty liver disease (NAFLD), the most common chronic liver diseases seen in gastroenterology clinics[3]. NAFLD represents a spectrum of disease, of which a subset with more severe liver disease; namely non-alcoholic steatohepatitis (NASH) may progress to cirrhosis[4]. Cirrhosis represents advanced stage liver disease where cumulative liver injury and necroinflammation result in fibrogenesis of the liver. This fibrogenesis is characterised by diffuse nodular regeneration, surrounded by dense fibrous septa, leading to parenchymal loss and collapse/distortion of normal liver architecture. Clinically, this correlates with the development of portal hypertension and hepatic synthetic dysfunction[5].

With increasing recognition of efficacy in treating obesity and obesity related diseases, bariatric surgery has been increasingly performed worldwide. Not unexpectantly, bariatric surgeons may encounter patients with NASH and NASH related cirrhosis, especially with the rising prevalence of obesity. A survey of bariatric surgeons found that 39% of them had unexpected discovery of cirrhosis during bariatric surgery, accounting for an incidence rate of 0.14%[6]. More importantly, the presence of cirrhosis often presents a vexing dilemma whether to proceed with surgery or not. This review explores the various important considerations in this context and suggests the best approaches to managing such patients.

CONSIDERATION OF LIVER FACTORS: SEVERITY OF CIRRHOSIS

Traditionally, surgical procedures in cirrhotic patients have been recognised to confer significant morbidity and mortality. Compared to non-cirrhotic patients undergoing surgery, patients with cirrhosis were reported to have longer length of hospital stay and higher hospitalisation charges[7]. Early studies report an overall 30% postoperative mortality and 11.6% thirty day postoperative mortality after various surgical procedures[8]. Further characterisation of surgical risk has identified severity of liver disease, nature and type of surgery to be important dependant factors. The Child-Turcotte-Pugh (CTP) score based on a combination of 5 clinical and laboratory parameters, correlates well with surgical risk and has been validated in several studies; in general, patients with Child's A cirrhosis have postoperative mortality rates of 10%, rising to 30% and 80% with Child's B and C, respectively[9-11]. One criticism of the CTP score is the subjective nature of assessing two components of the CTP score; ascites and encephalopathy, which may lead clinicians to under or over-estimate actual liver function[12]. The model for end stage liver disease (MELD) score, a more recently developed score, has also been shown to effectively predict postoperative mortality, in particular 30-d mortality, where a linear relationship is observed, with mortality rising 1% for each MELD point below 20 and 2% thereafter[13]. In general, a MELD score above 8 predicts a poor outcome[12]. The advantage of the MELD score is that it is less subjective than the CTP score as it utilises only objective laboratory parameters (serum bilirubin, international normalised ratio and creatinine). However, based on current literature, the superiority of one score over the other has not been clearly established[14]. In the setting of NASH, lifestyle modifications including sustained weight loss form the cornerstone of treatment. In this respect, bariatric surgery may provide the most consistent and effective method of achieving and maintaining adequate weight loss, as compared to exercise/diet and other lifestyle measures. In addition, the benefits of bariatric surgery in NASH may extend from direct effects of weight loss to indirect effects of improved insulin resistance and pro-inflammatory state[15]. Pooled data from a meta-analysis of 15 studies demonstrated improvement/resolution in steatosis (91.6%), steatohepatitis (81.3%) and fibrosis (65.5%)[16]. However, the current literature available consists only of data from cohort studies and not randomised controlled trials. Furthermore, most of the data pertained to subjects without advanced fibrosis/cirrhosis. As such, a 2010 Cochrane review was unable to provide any recommendations in this aspect[17]. Moreover, considering the invasive nature of bariatric surgery, there is an urgent need for well-designed randomised controlled studies to clarify the efficacy of such interventions in the treatment of NASH and specifically NASH cirrhosis.

CONSIDERATION OF LIVER FACTORS: PORTAL HYPERTENSION

Portal hypertension is another important factor to consider while assessing surgical risk. Portal hypertension is a frequent sequelae of cirrhosis, where fibrosis, scarring and the presence of regenerating nodules result in architectural distortion of the liver and in turn increased portal pressures[18]. Increased portal pressures contribute to the pathogenesis of many complications seen in cirrhosis, such as splenomegaly with thrombocytopenia, hyperkinetic circulatory state with ascites/unstable hemodynamics, gastro-eosophageal varices, hepatorenal syndrome and portopulmonary syndrome[19]. Intuitively, in the context of surgery, the thrombocytopenia connotes increased bleeding risk, presence of ascites may impact on healing and wound complications particularly in abdominal surgery, while also promoting atelectasis and pulmonary complications through reduced lung expansion. Perioperative hemodynamics and fluid balance may be difficult to manage. Perioperative developments of hepatorenal or portopulmonary syndromes compromise the renal and respiratory systems, making surgery all the more difficult and complicated.

The gold standard technique of assessing portal hypertension is using hepatic venous pressure gradient measurement (HVPG), which is a strong prognostic factor in cirrhosis. Clinically significant portal hypertension is observed when HVPG increased above 10 mmHg[18,20,21]. Other signs to suggest significant portal hypertension would include presence of gastro-oesophageal varices, splenomegaly with thrombocytopenia and presence of ascites. With respect to hepatic resection for hepatocellular carcinoma in cirrhotic patients, both European (European Association for the Study of the Liver) and American (American Association for the Study of Liver Diseases) liver society guidelines consider portal hypertension to be a relative contraindication for surgery[22,23], based on clinical studies observing increased risk of postoperative liver failure, complications and mortality in patients with portal hypertension compared to those without portal hypertension[24-26]. Similarly, in a nationwide population based American study on mortality following colorectal surgery, patients with cirrhosis and portal hypertension had significantly higher in-patient mortality compared to patients with cirrhosis but without portal hypertension (29% *vs* 14% mortality rate)[27]. This was consistent with a separate study exploring the impact of cirrhosis and portal hypertension on inpatient mortality across four specific types of surgery; cholecystectomy, colectomy, abdominal aortic aneurysm repair and coronary artery bypass grafting). Patients with cirrhosis and portal hypertension were at higher risk of mortality (Hazard ratio 7.8 to 22.7) compared to patients with cirrhosis alone (Hazard ratio 3.4 to 8) and patients without cirrhosis[7]. One method of addressing the issue of portal hypertension is through portal decompression using Transjugular Intrahepatic Portosystemic Shunt (TIPS) placement, which has been used to treat complications such as bleeding varices, refractory ascites, hepatic hydrothorax and hepatorenal syndromes[28]. Several small case series have suggested the potential role and benefits of TIPS placement in reducing perioperative complications and mortality rates across a variety of surgical procedures, showing one year survival ranging between 56% and 100%[29-32]. Having said that, the potential benefits of preoperative TIPS must be weighed against the risk of TIPS placement. Progressive hepatic failure is a rare but most feared complication post TIPS placement. It is often the reflection of decreased liver blood perfusion due to shunting of blood away from the liver, culminating in hepatic ischemia and progressive dysfunction in an already poorly functioning liver[33]. In addition, the incidence of new or worsening hepatic encephalopathy following TIPS is 20%-31%, which may limit the enthusiasm for this procedure[34,35]. Increasing age, liver dysfunction and shunt diameter are important risk factors associated with hepatic encephalopathy post-TIPS placement[36]. Hence, caution must be exercised when selecting appropriate patients to undergo a TIPS procedure[28].

In summary, common cirrhosis (including portal hypertension driven) related complications to be vigilant about in the post-operative setting would include worsening or new onset ascites, worsening or new onset hepatic encephalopathy, coagulopathy, fluid overload, renal failure or liver failure.

CONSIDERATION OF SURGICAL FACTORS

The nature and type of surgery impacts on the morbidity and mortality in cirrhotic patients. While postop mortality and morbidity rates have been high historically, better identification/awareness, preoperative assessment/optimisation, newer anaesthetics and surgical techniques have mitigated such risks. Overall, better outcomes are observed with laparoscopic surgery over open surgery. In a small case series of 50 predominantly mild to moderately cirrhotic patients undergoing a variety of laparoscopic surgical procedures, Cobb *et al*[37] found an overall morbidity rate of 16% with no incidence of hepatic decompensation or mortality. A separate population-based study advocated the preference of laparoscopic cholecystectomy over open cholecystectomy in cirrhotic patients, with improved mortality (1.3% *vs* 8.3%), less post-operative infection (0.7% *vs* 3.5%) and lower requirement for blood transfusion (14.4% *vs* 19.2%)[38]. In general, laparoscopic techniques have improved outcomes over a range of surgical procedures in patients with mild to moderate cirrhosis[37].

Separately, emergency surgery is generally regarded to have higher associated morbidity and mortality compared to elective surgery[39,40]. In addition, the surgical procedure itself has important implications and considerations in cirrhotic patients. Cardiac and major abdominal surgeries are considered high-risk surgeries in cirrhotic patients[14]. Assessing nationwide mortality rates

post elective surgery in cirrhotic patients, Csikesz *et al*[7] found disparate mortality rates among different surgical procedures (Hazard ratio 3.4, 3.7, 5.0 and 8.0 for cholecystectomy, colectomy, abdominal aortic aneurysm repair and coronary artery bypass grafting, respectively).

In summary, common surgical related complications among patients with cirrhosis would include sepsis, wound infection/dehiscence/impaired healing and bleeding.

IMPACT OF BARIATRIC SURGERY ON THE LIVER

There have been several case series on the impact of bariatric surgery on the liver. In the 1960s to early 1980s, the jejunoileal bypass (JIB) was among the first bariatric surgical procedures used to treat morbid obesity. However, this procedure has since been abandoned due to multiple side effects of this procedure, including the development of hepatic failure[41]. In a longitudinal follow up study of patients who had undergone JIB, the risk of development of hepatic fibrosis and cirrhosis at 15 years post JIB was 45% and 8%, respectively[42]. Several other studies have confirmed the risk of progressive liver dysfunction following JIB, where rapid weight loss, protein-caloric malnutrition, global malabsorption and endotoxin effects have all been implicated[43-45]. Hepatic decompensation (albeit inconsistent evidence) has also been reported following biliary pancreatic diversion and long limb Roux en Y gastric bypass[46-48]. There has been less evidence of detrimental effects on the liver following the other bariatric surgical procedures. As such, in a survey of 126 bariatric surgeon responders, 59% would perform banded gastroplasty, 39% standard Roux en Y gastric bypass and only 5% biliary pancreatic diversion in patients with cirrhosis[6]. Bariatric surgery may also be associated with nutritional deficiencies, depending on the type of procedure performed. In particular, protein deficiency remains a significant concern which can manifest as oedema, loss of lean muscle mass, as well as biochemical features of anaemia and hypoalbuminemia. Changes in taste/food preference, potential macronutrient mal-digestion or absorption contribute to reduced protein intake, coupled with the generally higher protein demand following surgery, leads to a compromised nitrogen balance and connotes an important problem[49,50]. This takes on even more consequence in the setting of cirrhosis, where pre-existing malnutrition, sarcopenia and impaired protein metabolism are prevalent[51]. Hence, the predicament being that cirrhotic patients may not be able to achieve adequate protein intake with the pre-existing cirrhosis and additional demands placed by the bariatric surgery.

REPORTED COMPLICATIONS OF BARIATRIC SURGERY IN PATIENTS WITH CIRRHOSIS

There have been relatively few studies describing complications of bariatric surgery in patients with cirrhosis (Table 1). In an early survey of 126 bariatric surgeons worldwide who had collectively performed almost 87000 bariatric surgeries, 125 cases of cirrhosis with overall incidence of 0.14% were unexpectedly encountered. While there was no intraoperative mortality observed, 4 deaths perioperatively (within 30 d) were related to overwhelming sepsis or fulminant hepatic failure. In addition, a further 7 late deaths were accounted for predominantly from liver failure. Separately, within the same paper, Brolin reported on 8 of his own patients with cirrhosis out of an overall series of 580 patients; hepatic failure accounted for 1 perioperative and 1 late mortality[6]. With the advent of laparoscopic approaches to bariatric surgery, surgical outcomes have improved further. Dallal *et al*[52] performed a retrospective review of 2119 patients who had underwent laparoscopic bariatric surgery and identified 30 patients with cirrhosis (1.4%), the majority of which were discovered intraoperatively. While there were no perioperative deaths, need for conversion to open laparotomy or liver related complications, early postoperative complications occurred in 9 of the cirrhotic patients, including anastomotic leak, acute tubular necrosis, prolonged intubation, ileus and blood transfusion. In addition, there were no major late complications other than 1 unrelated death from esophageal cancer one year post gastric bypass[52]. A subsequent case series of 23 cirrhotic patients who had undergone a combination of laparoscopic bariatric procedures (RYGB, sleeve gastrectomy, adjustable gastric banding) reported complications in 8 patients including anastomotic leak, structuring, infected hematoma, pneumonia and bleeding requiring blood transfusion. However, there was no liver decompensation after surgery and no perioperative mortality was reported. Of note, 2 of the cirrhotic patients successfully underwent laparoscopic sleeve gastrectomy after TIPS procedure to control their portal hypertension[53]. Takata *et al*[54] also advocated the safety and benefits of laparoscopic bariatric surgery in patients with cirrhosis; 2 out of 6 patients with cirrhosis who underwent laparoscopic sleeve gastrectomy had only minor complications (1 patient developed ascites that responded to medical therapy while the other patient developed hepatic encephalopathy secondary to urinary tract infection that also responded to standard therapy)[54]. Additional support for laparoscopic sleeve gastrectomy in cirrhotic patients was provided by Rebibo and colleages, who reviewed 13 patients with Child's A cirrhosis and observed no postoperative mortality or cirrhosis specific complications[55]. A unique complication of portal vein thrombosis with associated mesenteric ischaemia was described by Hughes and colleagues in a cirrhotic patient two weeks post laparoscopic sleeve gastrectomy. However, the patient recovered well post resection of her ischaemic bowel[56]. Further experiences of bariatric surgery in the context of cirrhosis patients were reported by Woodford *et al*[57], who reported two surgical complications, but no operative mortality in their series of 14 patients with compensated cirrhosis that underwent

Table 1 Study characteristics of bariatric surgery in patients with cirrhosis

Author, year	Number. cirrhotics	Childs status	Procedure	Average op time	Average blood loss	LOS	Complication	Mortality	Comments
Brolin 1998	8	NR	7 RYGB, 1 JIB reversal	NR	543 mL	NR	Ascitic fluid leak, marginal ulcer, amputation	1 periop death, 2 late deaths	
Dallal 2004	30	A	27 lap RYGB, 3 lap SG	4 h	290 mL	4	ATN, anastomotic leak, blood transfusion, prolonged ileus, prolonged intubation	1 late unrelated death	%EWL at 12 mo 63%
Takata 2008	6	4 A, 2B	6 Lap SG	141 min	58 mL	4.2	2 pts, development of ascites, HE post UTI	Nil	%EWL at 9 mo 33%
Shimizu 2013	23	22 A, 1B	14 lap RYGB, 8 lap SG, 1 LAGB	NR	NR	4.3	8 pts, anastomotic leak, stricture, infected hematoma, pneumonia	1 death 9 months postop of unknown cause	
Lin 2013	20	NR	Lap SG	151 min	NR	4.2	2 wound infections, 1 transient HE, 1 transient renal insufficiency, 1 bleeding, 1 staple line leak	1 death 4 years postop, 2 deaths on liver tx waiting list	
Rebibo 2014	13	13 A	13 lap SG	75 min	NR	3	1 postop intra-abdo hematoma	Nil	%EWL at 6 mo 61.9%
Hughes 2014	1	A	Lap SG	NR	NR	NR	Portal vein thrombosis with mesenteric ischaemia	Nil	
Woodford 2015	14	NR	14 LAGB	NR	NR	NR	1 postop surgical site infection, 1 revision of malpositioned band	1 unrelated death 11 yr post surg	%EWL at 12 mo 61.3%
Pestana 2015	14	A	11 SG, 3 RYGB	NR	NR	NR	1 unrelated HE 2 yr post-surgery	Nil	%Total weight loss at 24 mo 25.6%
Wolter 2016	12	NR	12 lap procedures	NR	NR	NR	1 staple line leak, 1 intra-abdominal abscess, 1 intraluminal bleed, 1 dysrhythmia	Nil	No significant association betw cirrhosis and periop events

NR: Not recorded; JIB: Jejunoileal bypass; SG: Sleeve gastrectomy; RYGB: Roux-en-Y gastric bypass; LAGB: Laparoscopic adjustable gastric banding; BPD: Biliopancreatic diversion; lap: Laparoscopic; %EWL: Percentage excess weight loss.

laparoscopic adjustable gastric banding. Comparable accounts were corroborated by a separate series of 14 cirrhotic patients that underwent sleeve gastrectomy or gastric bypass[58]. Along similar lines, Wolter *et al*[59] described 302 patients who had laparoscopic gastric bypass or sleeve gastrectomy performed, of which 12 patients had cirrhosis; there were no significant association between perioperative complications and liver cirrhosis. Nevertheless, in a large scale population based study of bariatric surgery patients, presence of compensated cirrhosis was associated with increased length of hospital stay (4.4 d *vs* 3.2 d, *P* = 0.03) and increased mortality rates (OR: 2.17, 95%CI: 1.03-4.55). Not unexpectedly, patients with decompensated cirrhosis had even worse outcomes. The combined in hospital mortality rate for both compensated and decompensated cirrhotics was 1.2%, with mortality rates lower at high volume centres (> 100 procedures per year) compared to lower volume centres[60]. More recently, in a retrospective study of 297 patients with NAFLD who had undergone bariatric surgery, while the median length of hospital stay was higher in patients with advanced liver fibrosis (4 d *vs* 3 d, *P* = 0.002) compared to those without advanced fibrosis, there was no significant difference in the proportion of patients with complications over 1 year post operation (36.4% *vs* 32.8%, *P* = 0.54) [61].

APPROACH TO BARIATRIC SURGERY IN PATIENTS WITH CIRRHOSIS

While the few case series on bariatric surgery in patients with cirrhosis do suggest that bariatric surgery can be performed safely in carefully selected patients without prohibitive complication rates, the majority of the studies were based on patients with well compensated cirrhosis. Therefore, it remains to be seen if such surgeries can be similarly performed successfully in patients with poorer hepatic reserves. Preferably, cirrhotic patients should undergo bariatric surgery in high volume centres

The first step is to recognise and diagnose patients with cirrhosis. This is usually based on biochemical and imaging features. Surgical risk can be further stratified using CTP or MELD score to estimate liver reserve. In addition, HVPG to assess degree of portal hypertension would be useful in further management. In the event of

significant portal hypertension, TIPS can be considered to reduce risk of perioperative complications[32]. Input from a hepatologist to optimise cirrhotic patients perioperatively would be valuable.

There remains no consensus on which bariatric modality is best suited for the patient with cirrhosis[62]. Current available data suggest that the less invasive laparoscopic approach would be safer to perform in cirrhotics. The bariatric procedures that can be performed laparoscopically would include the RYGB, sleeve gastrectomy and gastric banding, each representing their own distinct advantages and disadvantages.

In general, RYGP provides the most potential for weight loss, but may have a greater risk of vitamin deficiencies compared to sleeve gastrectomy that may further lead to progressive hepatic dysfunction. In addition, due to altered anatomy surgically, the stomach remnant and biliary tree would be inaccessible endoscopically in the event of a gastrointestinal bleed or biliary obstruction. Furthermore, the altered anatomy may render future orthotopic liver transplantation more challenging.

Gastric banding would be the least invasive procedure, but the potential risk of infection with placement of a foreign device in cirrhotic patients already at increased risk of infections would limit the enthusiasm of such a procedure.

Sleeve gastrectomy reduces the risk of malabsorption or placement of a foreign body, but may predispose to bleeding risk in the setting of gastric varices. Increasingly, laparoscopic sleeve gastrectomy has been advocated as the bariatric modality of choice in patients with cirrhosis[53-55]. Sleeve gastrectomy is well tolerated, technically less challenging with a relatively short learning curve and operating time[63]. Relative to gastric bypass, sleeve gastrectomy has been shown to be associated with fewer complications overall[64]. Furthermore, access to the stomach remnants and biliary system would remain possible with sleeve gastrectomy. With respect to liver transplantation, laparoscopic sleeve gastrectomy was observed to be well tolerated while improving candidacy for liver transplantation[54]. In addition, sleeve gastrectomy has been shown to be safe and feasible in combination with liver transplantation at a single setting or as a staged procedure post liver transplantation[65, 66].

Nevertheless, there remains important information gaps with regards to bariatric surgery in patients with cirrhosis. More studies are needed to assess the long term outcomes of patients with cirrhosis post bariatric surgery. In addition, more data is required in patients with more advanced cirrhosis. Better characterisation in terms of surgical candidacy, differentiating those who would derive benefit and those who would not, are imperative to develop strategies and recommendations for the improvement of bariatric surgical outcomes in patients with cirrhosis.

CONCLUSION

Bariatric surgery can be performed in patients with well compensated cirrhosis, typically of Child's A status, with minimal complication risks from surgical or hepatic factors. Patients need to be carefully selected and optimised, while surgical technique and modality also play equally important roles. With the onslaught of the NAFLD epidemic and anticipated increase in patients with NASH cirrhosis, bariatric surgery may provide an elixir as part of the armamentarium of therapeutic options.

REFERENCES

1   **Wang YC**, McPherson K, Marsh T, Gortmaker SL, Brown M. Health and economic burden of the projected obesity trends in the USA and the UK. *Lancet* 2011; **378**: 815-825 [PMID: 21872750 DOI: 10.1016/S0140-6736(11)60814-3]

2   **Kelly T**, Yang W, Chen CS, Reynolds K, He J. Global burden of obesity in 2005 and projections to 2030. *Int J Obes* (Lond) 2008; **32**: 1431-1437 [PMID: 18607383 DOI: 10.1038/ijo.2008.102]

3   **Younossi ZM**, Stepanova M, Afendy M, Fang Y, Younossi Y, Mir H, Srishord M. Changes in the prevalence of the most common causes of chronic liver diseases in the United States from 1988 to 2008. *Clin Gastroenterol Hepatol* 2011; **9**: 524-530.e1; quiz e60 [PMID: 21440669]

4   **Argo CK**, Caldwell SH. Epidemiology and natural history of non-alcoholic steatohepatitis. *Clin Liver Dis* 2009; **13**: 511-531 [PMID: 19818302 DOI: 10.1016/j.cld.2009.07.005]

5   **Tsochatzis EA**, Bosch J, Burroughs AK. Liver cirrhosis. *Lancet* 2014; **383**: 1749-1761 [PMID: 24480518 DOI: 10.1016/S0140-6736(14)60121-5]

6   **Brolin RE**, Bradley LJ, Taliwal RV. Unsuspected cirrhosis discovered during elective obesity operations. *Arch Surg* 1998; **133**: 84-88 [PMID: 9438765 DOI: 10.1001/archsurg.133.1.84]

7   **Csikesz NG**, Nguyen LN, Tseng JF, Shah SA. Nationwide volume and mortality after elective surgery in cirrhotic patients. *J Am Coll Surg* 2009; **208**: 96-103 [PMID: 19228510 DOI: 10.1016/j.jamcollsurg.2008.09.006]

8   **Ziser A**, Plevak DJ, Wiesner RH, Rakela J, Offord KP, Brown DL. Morbidity and mortality in cirrhotic patients undergoing anesthesia and surgery. *Anesthesiology* 1999; **90**: 42-53 [PMID: 9915311 DOI: 10.1097/00000542-199901000-00008]

9   **Garrison RN**, Cryer HM, Howard DA, Polk HC Jr. Clarification of risk factors for abdominal operations in patients with hepatic cirrhosis. *Ann Surg* 1984; **199**: 648-655 [PMID: 6732310 DOI: 10.1097/00000658-198406000-00003]

10 **Mansour A**, Watson W, Shayani V, Pickleman J. Abdominal operations in patients with cirrhosis: still a major surgical challenge. *Surgery* 1997; **122**: 730-5; discussion 735-6 [PMID: 9347849 DOI: 10.1016/S0039-6060(97)90080-5]

11 **Teh SH**, Nagorney DM, Stevens SR, Offord KP, Therneau TM, Plevak DJ, Talwalkar JA, Kim WR, Kamath PS. Risk factors for mortality after surgery in patients with cirrhosis. *Gastroenterology* 2007; **132**: 1261-1269 [PMID: 17408652 DOI: 10.1053/j.gastro.2007.01.040]

12 **Nicoll A**. Surgical risk in patients with cirrhosis. *J Gastroenterol Hepatol* 2012; **27**: 1569-1575 [PMID: 22694313 DOI: 10.1111/j.1440-1746.2012.07205.x]

13 **Northup PG**, Wanamaker RC, Lee VD, Adams RB, Berg CL. Model for End-Stage Liver Disease (MELD) predicts nontransplant surgical mortality in patients with cirrhosis. *Ann Surg* 2005; **242**: 244-251 [PMID: 16041215 DOI: 10.1097/01.sla.0000171327.29262.e0]

14 **Frye JW**, Perri RE. Perioperative risk assessment for patients with cirrhosis and liver disease. *Expert Rev Gastroenterol Hepatol* 2009; **3**: 65-75 [PMID: 19210114 DOI: 10.1586/17474124.3.1.65]

15 **Clanton J**, Subichin M. The Effects of Metabolic Surgery on Fatty Liver Disease and Nonalcoholic Steatohepatitis. *Surg Clin North Am* 2016; **96**: 703-715 [PMID: 27473796 DOI: 10.1016/j.suc.2016.03.008]

16 **Mummadi RR**, Kasturi KS, Chennareddygari S, Sood GK. Effect

of bariatric surgery on nonalcoholic fatty liver disease: systematic review and meta-analysis. *Clin Gastroenterol Hepatol* 2008; **6**: 1396-1402 [PMID: 18986848 DOI: 10.1016/j.cgh.2008.08.012]

17 **Chavez-Tapia NC**, Tellez-Avila FI, Barrientos-Gutierrez T, Mendez-Sanchez N, Lizardi-Cervera J, Uribe M. Bariatric surgery for non-alcoholic steatohepatitis in obese patients. *Cochrane Database Syst Rev* 2010; CD007340 [PMID: 20091629 DOI: 10.1002/14651858. CD007340.pub2]

18 **Berzigotti A**, Seijo S, Reverter E, Bosch J. Assessing portal hypertension in liver diseases. *Expert Rev Gastroenterol Hepatol* 2013; **7**: 141-155 [PMID: 23363263 DOI: 10.1586/egh.12.83]

19 **Nusrat S**, Khan MS, Fazili J, Madhoun MF. Cirrhosis and its complications: evidence based treatment. *World J Gastroenterol* 2014; **20**: 5442-5460 [PMID: 24833875 DOI: 10.3748/wjg.v20.i18.5442]

20 **Garcia-Tsao G**, Groszmann RJ, Fisher RL, Conn HO, Atterbury CE, Glickman M. Portal pressure, presence of gastroesophageal varices and variceal bleeding. *Hepatology* 1985; **5**: 419-424 [PMID: 3873388 DOI: 10.1002/hep.1840050313]

21 **Stanley AJ**, Robinson I, Forrest EH, Jones AL, Hayes PC. Haemodynamic parameters predicting variceal haemorrhage and survival in alcoholic cirrhosis. *QJM* 1998; **91**: 19-25 [PMID: 9519209 DOI: 10.1093/qjmed/91.1.19]

22 **Bruix J**, Sherman M; Practice Guidelines Committee, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma. *Hepatology* 2005; **42**: 1208-1236 [PMID: 16250051 DOI: 10.1002/hep.20933]

23 **European Association For The Study Of The Liver.**; European Organisation For Research And Treatment Of Cancer. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. *J Hepatol* 2012; **56**: 908-943 [PMID: 22424438 DOI: 10.1016/j.jhep.2011.12.001]

24 **Bruix J**, Castells A, Bosch J, Feu F, Fuster J, Garcia-Pagan JC, Visa J, Bru C, Rodés J. Surgical resection of hepatocellular carcinoma in cirrhotic patients: prognostic value of preoperative portal pressure. *Gastroenterology* 1996; **111**: 1018-1022 [PMID: 8831597 DOI: 10.1016/S0016-5085(96)70070-7]

25 **Llovet JM**, Fuster J, Bruix J. Intention-to-treat analysis of surgical treatment for early hepatocellular carcinoma: resection versus transplantation. *Hepatology* 1999; **30**: 1434-1440 [PMID: 10573522 DOI: 10.1002/hep.510300629]

26 **Poon RT**, Fan ST, Lo CM, Liu CL, Lam CM, Yuen WK, Yeung C, Wong J. Improving perioperative outcome expands the role of hepatectomy in management of benign and malignant hepatobiliary diseases: analysis of 1222 consecutive patients from a prospective database. *Ann Surg* 2004; **240**: 698-708; discussion 708-10 [PMID: 15383797 DOI: 10.1097/01.sla.0000141195.66155.0c]

27 **Nguyen GC**, Correia AJ, Thuluvath PJ. The impact of cirrhosis and portal hypertension on mortality following colorectal surgery: a nationwide, population-based study. *Dis Colon Rectum* 2009; **52**: 1367-1374 [PMID: 19617746 DOI: 10.1007/DCR.0b013e3181a80dca]

28 **Boyer TD**, Haskal ZJ; American Association for the Study of Liver Diseases. The role of transjugular intrahepatic portosystemic shunt in the management of portal hypertension. *Hepatology* 2005; **41**: 386-400 [PMID: 15660434 DOI: 10.1002/hep.20559]

29 **Schlenker C**, Johnson S, Trotter JF. Preoperative transjugular intrahepatic portosystemic shunt (TIPS) for cirrhotic patients undergoing abdominal and pelvic surgeries. *Surg Endosc* 2009; **23**: 1594-1598 [PMID: 19263108 DOI: 10.1007/s00464-009-0405-7]

30 **Azoulay D**, Buabse F, Damiano I, Smail A, Ichai P, Dannaoui M, Castaing D, Bismuth H. Neoadjuvant transjugular intrahepatic portosystemic shunt: a solution for extrahepatic abdominal operation in cirrhotic patients with severe portal hypertension. *J Am Coll Surg* 2001; **193**: 46-51 [PMID: 11442253 DOI: 10.1016/S1072-7515(01)00911-5]

31 **Gil A**, Martínez-Regueira F, Hernández-Lizoain JL, Pardo F, Olea JM, Bastarrika G, Cienfuegos JA, Bilbao JI. The role of transjugular intrahepatic portosystemic shunt prior to abdominal tumoral surgery in cirrhotic patients with portal hypertension. *Eur J Surg Oncol* 2004; **30**: 46-52 [PMID: 14736522 DOI: 10.1016/j.ejso.2003.10.014]

32 **Kim JJ**, Dasika NL, Yu E, Fontana RJ. Cirrhotic patients with a transjugular intrahepatic portosystemic shunt undergoing major extrahepatic surgery. *J Clin Gastroenterol* 2009; **43**: 574-579 [PMID: 19169145 DOI: 10.1097/MCG.0b013e31818738ef]

33 **Walser EM**, Nguyen M. Hepatic perfusion and hemodynamic effects of transjugular intrahepatic portosystemic shunts. *Semin Intervent Radiol* 2005; **22**: 271-277 [PMID: 21326705 DOI: 10.1055/s-2005-925553]

34 **Sanyal AJ**, Freedman AM, Shiffman ML, Purdum PP 3rd, Luketic VA, Cheatham AK. Portosystemic encephalopathy after transjugular intrahepatic portosystemic shunt: results of a prospective controlled study. *Hepatology* 1994; **20**: 46-55 [PMID: 8020904]

35 **Somberg KA**, Riegler JL, LaBerge JM, Doherty-Simor MM, Bachetti P, Roberts JP, Lake JR. Hepatic encephalopathy after transjugular intrahepatic portosystemic shunts: incidence and risk factors. *Am J Gastroenterol* 1995; **90**: 549-555 [PMID: 7717309]

36 **Riggio O**, Angeloni S, Salvatori FM, De Santis A, Cerini F, Farcomeni A, Attili AF, Merli M. Incidence, natural history, and risk factors of hepatic encephalopathy after transjugular intrahepatic portosystemic shunt with polytetrafluoroethylene-covered stent grafts. *Am J Gastroenterol* 2008; **103**: 2738-2746 [PMID: 18775022 DOI: 10.1111/j.1572-0241.2008.02102.x]

37 **Cobb WS**, Heniford BT, Burns JM, Carbonell AM, Matthews BD, Kercher KW. Cirrhosis is not a contraindication to laparoscopic surgery. *Surg Endosc* 2005; **19**: 418-423 [PMID: 15624057 DOI: 10.1007/s00464-004-8722-3]

38 **Chmielecki DK**, Hagopian EJ, Kuo YH, Kuo YL, Davis JM. Laparoscopic cholecystectomy is the preferred approach in cirrhosis: a nationwide, population-based study. *HPB* (Oxford) 2012; **14**: 848-853 [PMID: 23134187 DOI: 10.1111/j.1477-2574.2012.00562.x]

39 **Millwala F**, Nguyen GC, Thuluvath PJ. Outcomes of patients with cirrhosis undergoing non-hepatic surgery: risk assessment and management. *World J Gastroenterol* 2007; **13**: 4056-4063 [PMID: 17696222 DOI: 10.3748/wjg.v13.i30.4056]

40 **Carbonell AM**, Wolfe LG, DeMaria EJ. Poor outcomes in cirrhosis-associated hernia repair: a nationwide cohort study of 32,033 patients. *Hernia* 2005; **9**: 353-357 [PMID: 16132187 DOI: 10.1007/s10029-005-0022-x]

41 **Hocking MP**, Duerson MC, O'Leary JP, Woodward ER. Jejunoileal bypass for morbid obesity. Late follow-up in 100 cases. *N Engl J Med* 1983; **308**: 995-999 [PMID: 6835318 DOI: 10.1056/NEJM198304283081703]

42 **Hocking MP**, Davis GL, Franzini DA, Woodward ER. Long-term consequences after jejunoileal bypass for morbid obesity. *Dig Dis Sci* 1998; **43**: 2493-2499 [PMID: 9824141 DOI: 10.1023/A:1026698602714]

43 **O'Leary JP**. Hepatic complications of jejunoileal bypass. *Semin Liver Dis* 1983; **3**: 203-215 [PMID: 6353595 DOI: 10.1055/s-2008-1040686]

44 **Styblo T**, Martin S, Kaminski DL. The effects of reversal of jejunoileal bypass operations on hepatic triglyceride content and hepatic morphology. *Surgery* 1984; **96**: 632-641 [PMID: 6484807]

45 **Vyberg M**, Ravn V, Andersen B. Pattern of progression in liver injury following jejunoileal bypass for morbid obesity. *Liver* 1987; **7**: 271-276 [PMID: 3695814 DOI: 10.1111/j.1600-0676.1987.tb00355.x]

46 **Antal SC**. Prevention and Reversal of Liver Damage following Biliopancreatic Diversion for Obesity. *Obes Surg* 1994; **4**: 285-290 [PMID: 10742788 DOI: 10.1381/096089294765558520]

47 **Castillo J**, Fábrega E, Escalante CF, Sanjuan JC, Herrera L, Hernánz F, Martino E, Casafont F, Gómez Fleitas M. Liver transplantation in a case of steatohepatitis and subacute hepatic failure after biliopancreatic diversion for morbid obesity. *Obes Surg* 2001; **11**: 640-642 [PMID: 11594111 DOI: 10.1381/09608920160557174]

48 **Cotler SJ**, Vitello JM, Guzman G, Testa G, Benedetti E, Layden TJ. Hepatic decompensation after gastric bypass surgery for severe obesity. *Dig Dis Sci* 2004; **49**: 1563-1568 [PMID: 15573905 DOI: 10.1023/B:DDAS.0000043364.75898.c8]

49 **Moize V**, Geliebter A, Gluck ME, Yahav E, Lorence M, Colarusso T, Drake V, Flancbaum L. Obese patients have inadequate protein intake related to protein intolerance up to 1 year following Roux-

en-Y gastric bypass. *Obes Surg* 2003; **13**: 23-28 [PMID: 12630609 DOI: 10.1381/096089203321136548]

50   **Odstrcil EA**, Martinez JG, Santa Ana CA, Xue B, Schneider RE, Steffer KJ, Porter JL, Asplin J, Kuhn JA, Fordtran JS. The contribution of malabsorption to the reduction in net energy absorption after long-limb Roux-en-Y gastric bypass. *Am J Clin Nutr* 2010; **92**: 704-713 [PMID: 20739420 DOI: 10.3945/ajcn.2010.29870]

51   **Anand AC**. Nutrition and Muscle in Cirrhosis. *J Clin Exp Hepatol* 2017; **7**: 340-357 [PMID: 29234200 DOI: 10.1016/j.jceh.2017.11.001]

52   **Dallal RM**, Mattar SG, Lord JL, Watson AR, Cottam DR, Eid GM, Hamad G, Rabinovitz M, Schauer PR. Results of laparoscopic gastric bypass in patients with cirrhosis. *Obes Surg* 2004; **14**: 47-53 [PMID: 14980033 DOI: 10.1381/096089204772787284]

53   **Shimizu H**, Phuong V, Maia M, Kroh M, Chand B, Schauer PR, Brethauer SA. Bariatric surgery in patients with liver cirrhosis. *Surg Obes Relat Dis* 2013; **9**: 1-6 [PMID: 23201210 DOI: 10.1016/j.soard.2012.07.021]

54   **Takata MC**, Campos GM, Ciovica R, Rabl C, Rogers SJ, Cello JP, Ascher NL, Posselt AM. Laparoscopic bariatric surgery improves candidacy in morbidly obese patients awaiting transplantation. *Surg Obes Relat Dis* 2008; **4**: 159-164; discussion 164-165 [PMID: 18294923 DOI: 10.1016/j.soard.2007.12.009]

55   **Rebibo L**, Gerin O, Verhaeghe P, Dhahri A, Cosse C, Regimbeau JM. Laparoscopic sleeve gastrectomy in patients with NASH-related cirrhosis: a case-matched study. *Surg Obes Relat Dis* 2014; **10**: 405-410; quiz 565 [PMID: 24355322 DOI: 10.1016/j.soard.2013.09.015]

56   **Hughes DL**, Worrall SJ, Khan H, Cochrane R. Mesenteric ischaemia secondary to portomesenteric venous thrombosis, 2 weeks post laparoscopic sleeve gastrectomy in a cirrhotic patient. *BMJ Case Rep* 2014; **2014**: bcr2013202246 [PMID: 24618867]

57   **Woodford RM**, Burton PR, O'Brien PE, Laurie C, Brown WA. Laparoscopic Adjustable Gastric Banding In Patients with Unexpected Cirrhosis: Safety and Outcomes. *Obes Surg* 2015; **25**: 1858-1862 [PMID: 25708241 DOI: 10.1007/s11695-015-1623-9]

58   **Pestana L**, Swain J, Dierkhising R, Kendrick ML, Kamath PS, Watt KD. Bariatric surgery in patients with cirrhosis with and without portal hypertension: a single-center experience. *Mayo Clin Proc* 2015; **90**: 209-215 [PMID: 25659239 DOI: 10.1016/j.mayocp.2014.11.012]

59   **Wolter S**, Duprée A, Coelius C, El Gammal A, Kluwe J, Sauer N, Mann O. Influence of Liver Disease on Perioperative Outcome After Bariatric Surgery in a Northern German Cohort. *Obes Surg* 2017; **27**: 90-95 [PMID: 27272667 DOI: 10.1007/s11695-016-2253-6]

60   **Mosko JD**, Nguyen GC. Increased perioperative mortality following bariatric surgery among patients with cirrhosis. *Clin Gastroenterol Hepatol* 2011; **9**: 897-901 [PMID: 21782772 DOI: 10.1016/j.cgh.2011.07.007]

61   **Singh T**, Kochhar GS, Goh GB, Schauer P, Brethauer S, Kroh M, Aminian A, Lopez R, Dasarathy S, McCullough AJ. Safety and efficacy of bariatric surgery in patients with advanced fibrosis. *Int J Obes* (Lond) 2017; **41**: 443-449 [PMID: 27881858 DOI: 10.1038/ijo.2016.212]

62   **Wu R**, Ortiz J, Dallal R. Is bariatric surgery safe in cirrhotics? *Hepat Mon* 2013; **13**: e8536 [PMID: 23610589 DOI: 10.5812/hepatmon.8536]

63   **Trastulli S**, Desiderio J, Guarino S, Cirocchi R, Scalercio V, Noya G, Parisi A. Laparoscopic sleeve gastrectomy compared with other bariatric surgical procedures: a systematic review of randomized trials. *Surg Obes Relat Dis* 2013; **9**: 816-829 [PMID: 23993246 DOI: 10.1016/j.soard.2013.05.007]

64   **Li JF**, Lai DD, Ni B, Sun KX. Comparison of laparoscopic Roux-en-Y gastric bypass with laparoscopic sleeve gastrectomy for morbid obesity or type 2 diabetes mellitus: a meta-analysis of randomized controlled trials. *Can J Surg* 2013; **56**: E158-E164 [PMID: 24284156 DOI: 10.1503/cjs.026912]

65   **Heimbach JK**, Watt KD, Poterucha JJ, Ziller NF, Cecco SD, Charlton MR, Hay JE, Wiesner RH, Sanchez W, Rosen CB, Swain JM. Combined liver transplantation and gastric sleeve resection for patients with medically complicated obesity and end-stage liver disease. *Am J Transplant* 2013; **13**: 363-368 [PMID: 23137119 DOI: 10.1111/j.1600-6143.2012.04318.x]

66   **Lin MY**, Tavakol MM, Sarin A, Amirkiai SM, Rogers SJ, Carter JT, Posselt AM. Safety and feasibility of sleeve gastrectomy in morbidly obese patients following liver transplantation. *Surg Endosc* 2013; **27**: 81-85 [PMID: 22752278 DOI: 10.1007/s00464-012-2410-5]

ORIGINAL ARTICLE

Basic Study

Impact of hyperglycemia on autoimmune pancreatitis and regulatory T-cells

Franz-Tassilo Müller-Graff, Brit Fitzner, Robert Jaster, Brigitte Vollmar, Dietmar Zechner

Franz-Tassilo Müller-Graff, Brigitte Vollmar, Dietmar Zechner, Institute for Experimental Surgery, Rostock University Medical Center, Rostock 18057, Germany

Brit Fitzner, Robert Jaster, Division of Gastroenterology, Department of Medicine Ⅱ, Rostock University Medical Center, Rostock 18057, Germany

ORCID number: Franz-Tassilo Müller-Graff (0000-0002-0566-9278); Brit Fitzner (0000-0003-3077-1351); Robert Jaster (0000-0002-8220-4570); Brigitte Vollmar (0000-0002-2403-9597); Dietmar Zechner (0000-0002-2075-7540).

Abstract

AIM
To evaluate the influence of hyperglycemia on the progression of autoimmune pancreatitis.

METHODS
We induced hyperglycemia by repetitive intraperitoneal (ip) injection of 50 mg/kg streptozotocin in MRL/MpJ mice, which develop autoimmune pancreatitis due to a genetic predisposition. We compared the extent of inflammation (histological score, CD3$^+$ lymphocytes, CD8$^+$ T-cells, CD4$^+$ T-cells, Foxp3$^+$ T-helper cells) in the pancreas of hyperglycemic and normoglycemic mice. We also analyzed the number of leukocytes, lymphocytes, granulocytes and monocytes in the blood. In addition, we determined the percentage of CD3$^+$ lymphocytes, CD8$^+$ T-cells, CD4$^+$ T-cells, Foxp3$^+$ T-helper cells, Foxp3$^+$ CD25$^+$ T-helper and Foxp3$^-$ T-helper cells in the spleen by flow cytometry.

RESULTS

Treatment with streptozotocin caused a strong induction of hyperglycemia and a reduction in body weight ($P < 0.001$). Severe hyperglycemia did not, however, lead to an aggravation, but rather to a slight attenuation of autoimmune pancreatitis. In the pancreas, both the histological score of the pancreas as well as the number of CD3^{+} lymphocytes ($P < 0.053$) were decreased by hyperglycemia. No major changes in the percentage of CD8^{+} T-cells, CD4^{+} T-cells, Foxp3^{+} T-helper cells were observed between hyperglycemic and normoglycemic mice. Hyperglycemia increased the numbers of leukocytes ($P < 0.001$), lymphocytes ($P = 0.016$), granulocytes and monocytes ($P = 0.001$) in the blood. Hyperglycemia also moderately reduced the percentage of CD3^{+} lymphocytes ($P = 0.057$), significantly increased the percentage of Foxp3^{+} T-helper cells ($P = 0.018$) and Foxp3^{+} CD25^{+} T-helper cells ($P = 0.021$) and reduced the percentage of Foxp3^{-} T-helper cells ($P = 0.034$) in the spleen.

CONCLUSION

Hyperglycemia does not aggravate but moderately attenuates autoimmune pancreatitis, possibly by increasing the percentage of regulatory T-cells in the spleen.

Key words: Autoimmune disease; Diabetes; Treg; FoxP3; Autoimmune pancreatitis; MRL/MpJ mice

Core tip: Temporary or sustained hyperglycemia can be observed in about 42%-66% of patients with autoimmune pancreatitis. However, it is unknown to what extent hyperglycemia has an influence on the course of this disease. This preclinical study demonstrates that hyperglycemia does not lead to an aggravation but rather an attenuation of autoimmune pancreatitis. Thus, this result might have the clinical implication that a tight adjustment of blood glucose concentration in patients with autoimmune pancreatitis is not needed, because it might not have a beneficial effect on the progression of this disease.

INTRODUCTION

Autoimmune pancreatitis (AIP) is a chronic and progressive inflammatory disease of the pancreas with an assumed autoimmune etiology, which was first described by Sarles *et al*[1] in 1961. The disease is classified by two different histopathological patterns called lympho-plasmacytic sclerosing pancreatitis (LPSP; AIP type 1) and idiopathic duct centric pancreatitis (ICDP; AIP type 2). AIP presents as a versatile disease with cholestasis, obstructive jaundice and pancreatic swelling. Besides the lymphoplasmacytic infiltrate, fibrosis is often pronounced, and a strong response to steroids can be seen. Besides these similarities, the two types differ in other characteristics such as serological markers, in particular elevated levels of IgG4 in LPSP, other organ involvement, or disease relapse[2-4]. The differentiation to pancreatic neoplasia is difficult, which sometimes can lead to unnecessary pancreatectomy[5].

The inflammatory infiltrate in the pancreas during AIP is mainly composed of lymphocytes and plasma cells as well as some eosinophil and neutrophil granulocytes and dendritic cells. Frequently, T-cells are observed, in particular CD4^{+}-T-helper cells and CD8^{+} cytotoxic cells[6]. In addition, regulatory T cells (Tregs) can be found, which are relevant for the immunological self-tolerance by suppressing autoreactive lymphocytes[7]. A specific marker for these cells is the transcription factor forkhead-box-protein P3 (FoxP3), which has a major influence on the development of regulatory T-cells[8,9]. Increased numbers of these cells were found locally in the pancreas as well as in the peripheral blood during AIP[7,10].

A frequent and important complication of AIP is diabetes mellitus (DM). The rate of DM in patients with AIP varies in the literature between 42%-66.7%[11,12]. Of those, 34%-43% already have DM before AIP, 52%-57% show a simultaneous occurrence and 9%-14% develop DM after starting a steroid therapy[13-15]. These findings suggest that both pathologies are present simultaneously in many patients. A correlation between hyperglycemia and pancreatitis in patients is well recognized, which is in general explained by the fact that pancreatitis can trigger DM[16,17]. Investigations into whether DM or transient hyperglycemia can enhance the development of a pancreatitis in return are rare. Previous preclinical studies demonstrate that diabetes dramatically aggravates the course of cerulein-induced acute and chronic pancreatitis[18,19]. Thus, the purpose of this present study was to address the question of whether diabetes can influence the progression of AIP and to analyze which aspects of this disease are affected by diabetes. We chose to use MRL/MpJ mice, an animal model widely used to study AIP. These mice spontaneously develop AIP with an incidence of 74% in female and 36% in male animals at the age of 34-38 wk[20].

MATERIALS AND METHODS

Animals

All mice were bred in the local SPF facility (the health of the animal stock is routinely checked according to FELASA guidelines) and were exposed to a 12 h artificial light/dark cycle. The mice were caged in groups and were allowed access to water and standard laboratory chow *ad libitum*. Only female MRL/MpJ mice of the

same age (Sham: 210/204-260, STZ: 210/204-224 median/interquartile range in days) were either sham- or streptozotocin-treated (STZ) in the laboratory. Hyperglycemia was induced by intraperitoneal (ip) injection (between 8:00 and 18:00) of 50 mg/kg STZ (Sigma-Aldrich, Steinheim, Germany) on five consecutive days (day 1-5). All control mice were sham-treated with 50 mmol/L sodium citrate pH 4.5 instead of STZ. Animals were allocated in a non-random manner matching the age of both treatment groups. Tissue was harvested on day 113-116 after first STZ injection. For sampling blood and tissue at the indicated time points the animals were anesthetized (by ip injection) with 90 mg/kg ketamine (Bela-Pharm, Vechta, Germany) and 8 mg/kg xylazine (Bayer Health Care, Leverkusen, Germany). The order in which animals were treated and blood and tissue was sampled was arbitrary. All experiments were executed in accordance with the German legislation, the EU-directive 2010/63/EU and approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern.

Histological and immunofluorescence staining

On the day of tissue harvesting, the rostral half of the pancreas was placed lengthwise in a biopsy cassette, fixed in 4% phosphate-buffered formalin, and embedded in paraffin. The second half was embedded in Tissue-Tek OCT Compound (Science Services GmbH, Munich, Germany). Paraffin sections of 4 μm thickness were cut and stained with hematoxylin and eosin to evaluate the organ. The histology of the pancreas was investigated by light microscopy (Olympus Corporation, Tokyo, Japan). The severity of autoimmune pancreatitis was determined by scoring the degree of inflammatory cell infiltration and destruction of the parenchyma as described by Kanno *et al*[20] (0 = no inflammation, healthy organ; 1 = mild inflammation, mononuclear cells present in the interstitium but no destruction of parenchyma; 2 = moderate inflammation, focal destruction of parenchyma with mononuclear cell infiltration; 3 = moderate and diffuse or severe but focal inflammation, diffuse destruction of parenchyma with residues of intact parenchyma; 4 = severe and diffuse inflammation, extended mononuclear cell infiltrates with destruction of acini and replacement by adipose tissue)[20,21]. The score of two to three sections was averaged to receive a final score by at least two independent reviewers in a blinded manner. In case of discrepancies, a third investigator was consulted for a joint review.

Immunofluorescence was performed using the antibodies eFluor660 conjugated rat anti-CD3 (50-0032; dilution: 600x; eBioscience, Frankfurt, Germany), FITC conjugated rat anti-CD4 (11-0041; dilution: 1200 ×; eBioscience), FITC conjugated rat anti-CD8 (ab25676; dilution: 200 ×; Abcam, Cambridge, United Kingdom) and rabbit anti-FoxP3 (ab54501; dilution: 2100 ×; Abcam) with subsequent secondary antibody Dylight594 goat-anti-rabbit (Dianova, Hamburg, Germany). Immuno-

fluorescence was evaluated on scanned sections with a Leica DMI 4000B scanning microscope using a 40 × objective (Leica Microsystems GmbH, Wetzlar, Germany).

Flow cytometry

To evaluate subtypes of T-cells, single-cell suspensions were isolated from the spleen of STZ- and sham-treated mice. A total of 1×10^6 leukocytes were stained in a volume of 100 μL PBS pH 7.4 (10010-015, Thermo Fisher Scientific GmbH) containing 0.1% BSA (A9418-10G, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and appropriate antibodies. These antibodies were eFluor660 conjugated rat anti CD3 (50-0032; dilution: 100 ×; eBioscience, Frankfurt, Germany), FITC conjugated rat anti-CD4 (11-0041; dilution, 200 ×; eBioscience), PE conjugated rat anti-CD8a (BD Cat 553032; dilution: 100 ×; BD Bioscience, Heidelberg, Germany) and PE-Cy7 conjugated rat anti-CD25 (25-0251; dilution: 200 ×; eBioscience). Regulatory T-cells were evaluated by staining with FITC conjugated rat anti CD4 and PE conjugated mouse anti-FOXP3 (130-093-014; dilution, 10 ×; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Data were recorded using a BD FACSCalibur and analyzed using the BD CellQuest Pro software (both Becton Dickinson, NJ, United States).

Analysis of the blood

Progression of hyperglycemia was monitored with the blood glucose meter Contour (Bayer Vital, Leverkusen, Germany). A differential blood cell count was performed with the automated hematology analyzer Sysmex KX 21 (Sysmex Cooperation, Kobe, Japan). To assess acinar cell damage, lipase and amylase activity in blood plasma was analyzed using the Cobas c111 spectrophometer (Roche Diagnostics, Mannheim, Germany).

Statistical analysis

Data are presented as box plots indicating the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles as whiskers. Graphs and statistics were made by using SigmaPlot software version 12.0. Three independent replications (always containing animals of both treatment groups) were done for each experiment. The statistical methods of this study were reviewed by Dr. Änne Glass of the Rostock University Medical Center.

The primary outcome was defined at the beginning of the study as number of T-Lymphocytes per field in the pancreas. No sample size calculation was used. Mice that did not develop hyperglycemia after treatment with STZ and mice that died during this long term experiment (an identical number of mice in both treatment groups) were excluded from the analysis. For data acquisition and description, the ARRIVE guidelines were respected. The significance of differences was evaluated using a Mann Whitney rank-sum test. Differences with $P \leqslant 0.05$ were considered to be significant. Differences with $P \leqslant 0.08$ were considered to indicate a tendency.
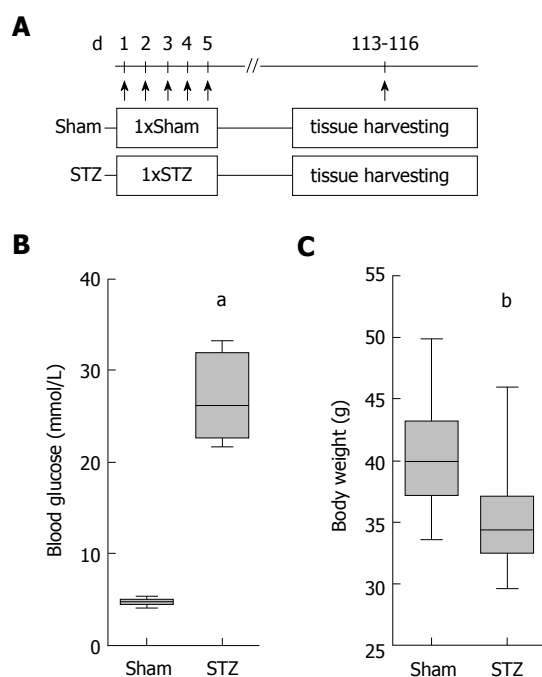
A

B

C

Figure 1 Experimental protocol of the hyperglycemic autoimmune pancreatitis model. 28-40 wk old- MRL/MpJ mice were intraperitoneally (ip) injected with streptozotocin on day 1-5 (group: STZ), while one age-matched control cohort was ip injected with the appropriate vehicle (group: Sham). The tissue was harvested on day 113-116; (A) Treatment with STZ increased the blood glucose concentration; (B) and reduced the body weight on day 113-116; (C) Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles as whiskers. The number of animals evaluated was n = 19 (Sham) and n = 17 (STZ). Differences between the indicated cohorts are indicated as significant. [a]$P < 0.001$; [b]$P = 0.009$. STZ: Streptozotocin-treated.

RESULTS

Influence of hyperglycemia on the course of autoimmune pancreatitis

In order to evaluate the influence of hyperglycemia on the course of AIP, we used MRL/MpJ mice due to their spontaneous development of AIP. Distinct cohorts were sham-treated or, in order to induce hyperglycemia, ip injected with STZ for five days and the pancreas was evaluated on day 113-116 (Figure 1A). STZ was effective since it increased the blood glucose concentration on day 22 (Sham: 4.6/4.1-5.5 median/interquartile range in mmol/L. STZ: 22.1/18.2-25.5 median/interquartile range in mmol/L) until the end of the experiment (Figure 1B) and reduced the body weight (Figure 1C). To investigate the severity of AIP, a histological score with numbers from zero to four was used (Figure 2A). Thirteen weeks of hyperglycemia caused a slight decrease in the severity of the histological score and therefore a moderate, non-significant, improvement of AIP when compared to normoglycemic mice (Figure 2B). Little difference was seen in the pancreas to body weight ratio (Figure 2C). Hyperglycemia did not increase the lipase (Figure 2D) or the amylase activity (Figure 2E).

Evaluation of local pancreatic inflammation during autoimmune pancreatitis

To evaluate the composition of the inflammatory infiltrates in the pancreas, we differentiated between T-lymphocytes (CD3$^+$ cells), T-helper cells (CD3$^+$CD4$^+$cells) and cytotoxic T-cells (CD3$^+$CD8$^+$ cells) by immunofluorescence staining (Figure 3A and B). T-Lymphocytes (defined as number of CD3$^+$ per field) were decreased in diabetic mice compared to non-diabetic mice (Figure 3C). STZ had almost no influence on the percentage of cytotoxic T-cells (CD8$^+$CD3$^+$ cells $\times$ 100 divided by the number of CD3$^+$cells) (Figure 3D), the percentage of T-helper cells (CD4$^+$CD3$^+$ cells $\times$ 100 divided by the number of CD3$^+$cells) (Figure 3E) and the percentage of regulatory T-cells (FoxP3$^+$CD4$^+$cells $\times$ 100 divided by the number of CD4$^+$ cells) (Figure 3F). Thus, no increase, but rather a reduction in the number of CD3+ leukocytes, was observed in the pancreas of hyperglycemic mice.

Influence of hyperglycemia on leukocytes during autoimmune pancreatitis

To analyze the systemic effect of hyperglycemia during AIP, we sampled blood on the day of tissue harvesting. A significant increase in the number of leukocytes (Figure 4A), lymphocytes (Figure 4B), as well as granulocytes plus monocytes (Figure 4C), was observed in hyperglycemic mice compared to normoglycemic animals.

Since these major differences in the number of immune cells were seen in the blood, we investigated whether hyperglycemia also had an influence on immune cells in the spleen. For this purpose, we analyzed the splenic cells by fluorescent activated cell sorting, which visualized lymphocytes, their stained subpopulations of T-helper cells (CD3$^+$CD4$^+$ cells) and cytotoxic T-cells (CD3$^+$CD8$^+$ cells) as well as regulatory T-cells (FoxP3$^+$CD4$^+$cells) (data not shown and Figure 5A). The quantification of these cells showed a tendentious decrease in the percentage of T-lymphocytes (defined as number of CD3$^+$ cells $\times$ 100 divided by the number of lymphocytes) in mice treated with STZ compared to controls (Figure 5B). STZ also modestly increased the percentage of cytotoxic T-cells (CD8$^+$CD3$^+$ cells $\times$ 100 divided by the number of CD3$^+$cells) (Figure 5C), and reduced those of T-helper cells (CD4$^+$CD3$^+$ cells $\times$ 100 divided by the number of CD3$^+$cells) (Figure 5D). Hyperglycemia significantly increased the percentage of regulatory T-cells (FoxP3$^+$CD4$^+$cells $\times$ 100 divided by the number of CD4$^+$ cells) (Figure 5E). Interestingly, hyperglycemia also significantly increased the percentage of CD25 expressing regulatory T-cells (FoxP3$^+$ CD25$^+$ CD4$^+$ cells v 100 divided by the number of CD4$^+$cells) (Figure 5F) compared to normoglycemic mice, whereas the percentage of FoxP3$^-$ T-cells (FoxP3$^-$CD4$^+$cells $\times$ 100 divided by the number of CD4$^+$ cells) was significantly reduced in STZ-treated mice (Figure 5G). Thus, a major influence of hyperglycemia on regulatory T-cells was
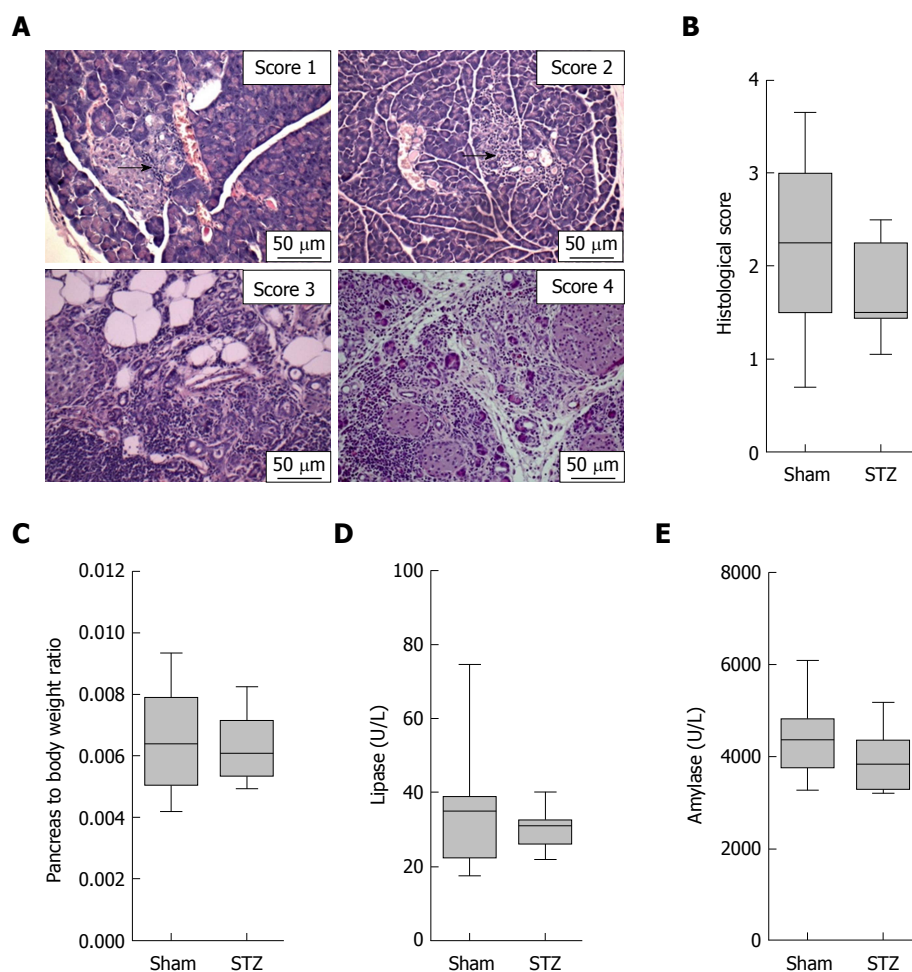
Figure 2 Evaluation of autoimmune pancreatitis. A: Representative images of the histological scores of autoimmune pancreatitis. Arrows point at small inflammatory infiltrates embedded in healthy surrounding tissue; B: hyperglycemia (STZ) reduced the histological score slightly compared to normoglycemic controls (sham); little differences are observed in the pancreas to body weight ratio (C), lipase activity (D), and amylase activity (E). The number of animals evaluated was n = 19 (sham) and n = 17 (STZ) in Panel B and C and n = 19 (Sham) and n = 15 (STZ) in D and E. Scale bar = 50 μm. STZ: Streptozotocin-treated.

observed in the spleen.

DISCUSSION

In order to test the influence of hyperglycemia on the course of AIP, we compared the progression of AIP in diabetic and non-diabetic mice. Surprisingly, experimental hyperglycemia didn't cause an aggravation of AIP in MRL/MpJ mice, but moderately improved autoimmune pancreatitis (Figures 2B and 3C). This is in contrast to our previous studies, where hyperglycemia lead to a major aggravation of cerulein-induced acute and chronic pancreatitis[18,19]. This might be explained by the differences in the pathogenesis of these two distinct types of pancreatitis. It is currently controversial whether cerulein-induced pancreatitis is mediated by premature activation of trypsinogen or by persistent activation of the NF-κB pathway[22,23]. In contrast, AIP is suggested to be caused by an autoimmunological event, which leads to inflammatory infiltration of the organ[1-4]. A beneficial effect of hyperglycemia on autoimmune pancreatitis seems to be counterintuitive, since hyperglycemia has been demonstrated to have a deleterious effect on many

organs. However, hyperglycemia can also have an inhibitory effect on the immune system. One could speculate that this impact on the immune system may have a beneficial effect on the progression of some autoimmune diseases.

Our study has several limitations. For example, we cannot completely exclude that instead of high glucose concentration in the blood, the drug we used for inducing hyperglycemia (STZ) influences AIP directly. However, in our opinion it is very unlikely that the alkylating agent STZ, which has been demonstrated to be cytotoxic to cells, directly cures AIP. We also limited our study to evaluate the effect of sustained hyperglycemia on AIP and can therefore not conclude if a transient hyperglycemia also has beneficial effects on the progression of AIP. Another limitation of this study is the use of a histological score, which was defined by the ordinal numbers 0 to 4. Comparing the median of the histological score allows, therefore, only a restricted presentation of complex changes in the histology of the pancreas[20]. We observed that the median of this score was reduced from 2.25 in normoglycemic to 1.50 in hyperglycemic mice (Figure 2B). However, this simple comparison underestimates the
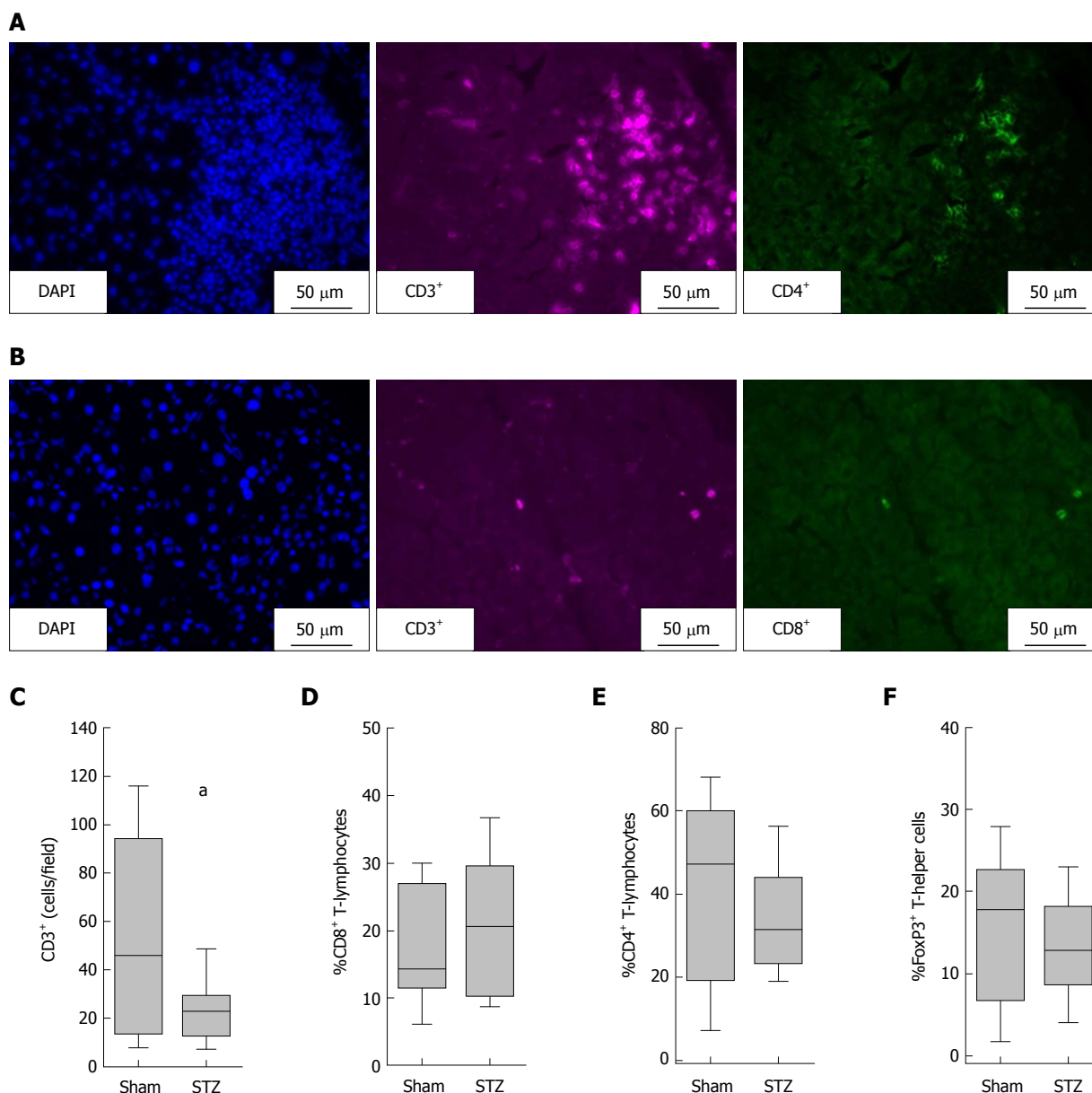
Figure 3 Evaluation of the local pancreatic inflammation during autoimmune pancreatitis. Representative images of T-helper cells (CD3+CD4+cells) (A); and cytotoxic T-cells (CD3+CD8+ cells) (B); hyperglycemia induced by STZ decreased the number of T-lymphocytes (defined as number of CD3+ per field) (C); little differences are observed in the percentage of cytotoxic T-lymphocytes (CD8+CD3+ cells × 100 divided by the number of CD3+cells) (D); T-helper cells (CD4+CD3+ cells × 100 divided by the number of CD3+cells) (E); and regulatory T-cells (FoxP3+CD4+cells × 100 divided by the number of CD4+ cells) (F). The number of animals evaluated was *n* = 19 (Sham) and *n* = 17 (STZ). Differences between the indicated cohorts are indicated as tendency, [a]*P* = 0.053. STZ: Streptozotocin-treated.

observed changes. The same data can also be presented as the percentage of mice with a histological score of ⩾ 3. Under normoglycemic conditions, 42% of mice (8 from 19 mice) received a histological score of ⩾ 3. Under hyperglycemic conditions, only 6% (1 from 17 mice) received a score of ⩾ 3. These high scores were assigned when moderate diffuse or severe focal inflammation plus a diffuse destruction of the acini (score 3) or severe and diffuse inflammation plus extended destruction of acini (score 4) was observed[20,21]. This impressive difference in the percentage of mice with a high histological score suggests that hyperglycemia reduces the damage to the parenchyma, which might indirectly reduce local inflammation. Alternatively, this observation could also

suggest that hyperglycemia reduces inflammation and thus minimizes the damage to the parenchyma. Due to the following observations, we prefer the second option. We found increased numbers of regulatory T-cells and decreased FoxP3- T-cells in the spleen. This is consistent with data in another preclinical study, where it has been described, that decreased numbers of regulatory T-cells and increased numbers of FoxP3- T-cells in the spleen lead to an increased severity of AIP[24]. In both studies, the numbers of regulatory T-cells and FoxP3- T-cells in the spleen correlate with the severity of AIP. Indeed, one study demonstrated a suppressive effect of transferred Tregs on autoimmune pancreatitis in MRL/MpJ mice[25]. However, the observed reduction in the histological score
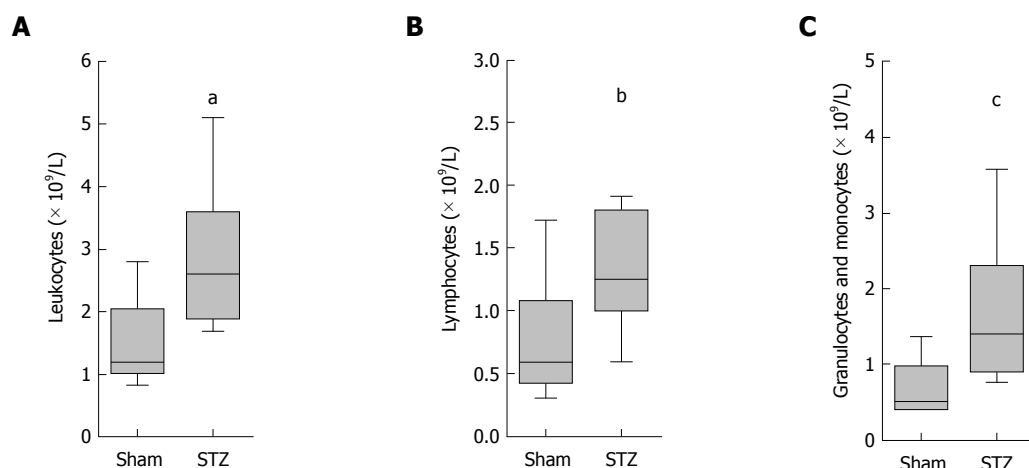
Figure 4 Diabetes increased the number of leukocytes in the blood. Treatment with STZ raised the number of leukocytes (A); lymphocytes (B); as well as granulocytes and monocytes (C). The number of animals evaluated was *n* = 19 (Sham) and *n* = 14 (STZ). Differences between the indicated cohorts are indicated as significant, (A) [a]*P* < 0.001; (B) [b]*P* = 0.016; (C) [c]*P* = 0.001. STZ: Streptozotocin-treated.

was not significant[25].

We observed major differences between hypergly-cemic and normoglycemic mice when analyzing immune cells in the blood and the spleen in MRL/MpJ mice. We noticed (1) increased percentage of regulatory T-cells and (2) decreased percentage of FoxP3- T-cells in the spleen, as well as an (3) increased number of leukocytes in the blood of STZ-treated mice (Figures 4A-C and 5E-G). These findings suggest that hyperglycemia might rather influence the overall immune system and only in-directly affect AIP itself. This interpretation is consistent with preclinical studies, which analyzed the influence of hyperglycemia on regulatory T-cells in mice not suffering from AIP. In these animals, hyperglycemia also increased the number of CD4+CD25+Foxp3+ regulatory T-cells in lymph nodes and the spleen of mice[26,27].

For treating AIP in the clinic, guidelines recommend to adjust blood glucose concentration before starting a steroid therapy[28,29]. This is mainly based on publications suggesting that a preexisting DM before the onset of AIP is worsening in 75% of patients after the start of a steroid therapy[28,29]. However, our preclinical study shows that hyperglycemia does not have a negative influence on AIP. Critical attitudes towards adjusting the blood glucose concentration in patients suffering from AIP have been previously published. For example, in one third of all cases, DM even worsened after insulin therapy[15]. Moreover, it was reported that treatment of diabetes with insulin led to hypoglycemic attacks in 10 from 50 AIP patients[13]. In addition, several clinical studies could demonstrate that DM improved in many cases imme-diately after steroid therapy[15,30]. These clinical studies argue against a tight control of blood glucose in AIP patients. Although our study has the limitation to be only a preclinical study on mice, it also suggests that an ag-gressive adjustment of blood glucose concentration might not be necessary as a prerequisite for the treatment of AIP. For final clarification of this issue, a clinical study evaluating if a tight adjustment of blood glucose in addi-tion to steroid therapy is beneficial or harmful to patients with AIP might need to be pursued.

ARTICLE HIGHLIGHTS

Research background
In about 42%-66% of patients with autoimmune pancreatitis, hyperglycemia can be observed. Thus, hyperglycemia is a frequent and important complication of this disease. However, it is not known if it merely reflects the severity of pancreatitis or whether it can also influence the progression of autoimmune pancreatitis.

Research motivation
For treating autoimmune pancreatitis in the clinic, guidelines recommend to adjust blood glucose concentration before starting a steroid therapy. However, critical attitudes towards adjusting the blood glucose concentration in these patients have also been published. For example, in one third of all cases, hyperglycemia even worsened after insulin therapy, and treatment of diabetes with insulin sometimes leads to hypoglycemic attacks in patients. In order to decide how important a tight adjustment of blood glucose concentration in patients with autoimmune pancreatitis is, we need to know if hyperglycemia has a positive or negative influence on the progression of this disease.

Research objectives
The purpose of this present study was to address the question of whether diabetes can influence the progression of autoimmune pancreatitis and to analyze which aspects of this disease are affected by hyperglycemia.

Research methods
We chose to use MRL/MpJ mice, an animal model widely used to study autoimmune pancreatitis. These mice spontaneously develop autoimmune pancreatitis. We induced hyperglycemia by repetitive intraperitoneal (ip) injection of streptozotocin in female mice and compared the extent of inflammation in the pancreas of hyperglycemic and normoglycemic animals. We also analyzed the number of immune cells in the blood. In addition, we determined the percentage of T-cells, especially regulatory T-cells in the spleen.

Research results
Surprisingly, experimental hyperglycemia did not cause an aggravation of autoimmune pancreatitis, but moderately improved autoimmune pancreatitis. We noticed an increased percentage of regulatory T-cells in the spleen, as well as an increased number of leukocytes in the blood of hyperglycemic mice. These findings suggest that hyperglycemia might rather influence the overall immune system and only indirectly affect autoimmune pancreatitis itself.
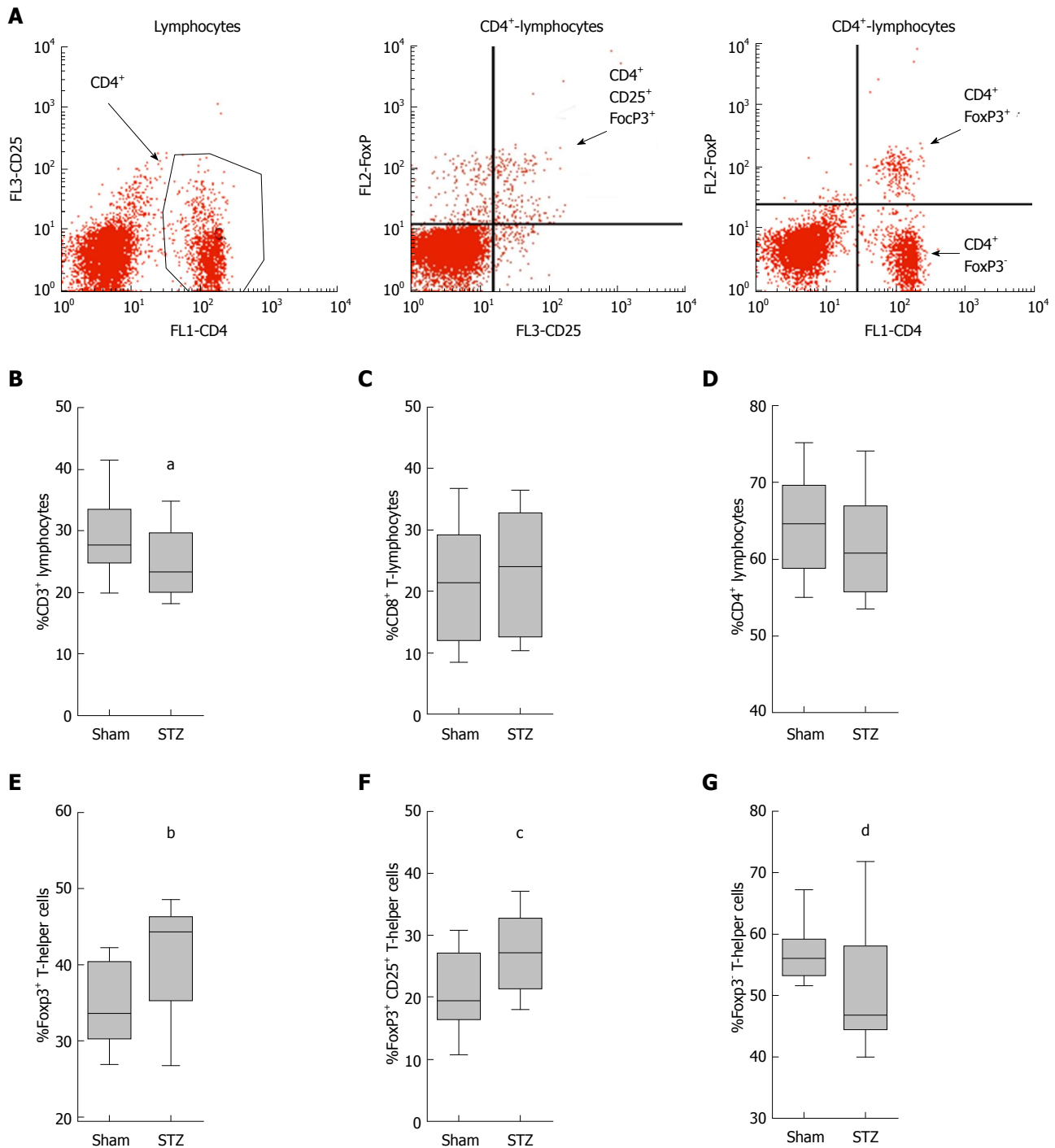
Figure 5 Evaluation of leukocytes in the spleen during autoimmune pancreatitis. Representative images of stained T-helper cells and their distinction between regulatory T-cells (FoxP3+CD4+ cells), CD25 expressing regulatory T-cells (CD4+CD25+ FoxP3+ cells) and FoxP3- T-cells (FoxP3- CD4+ cells) (A); application of STZ decreased the percentage of T-lymphocytes (defined as number of CD3+ cells × 100 divided by the number of lymphocytes) (B); little differences are observed in the percentage of cytotoxic T-cells (CD8+CD3+ cells × 100 divided by the number of CD3+cells) (C); and T-helper cells (CD4+CD3+ cells × 100 divided by the number of CD3+cells) (D); STZ increased the percentage of regulatory T-cells (FoxP3+CD4+cells × 100 divided by the number of CD4+ cells) (E); and CD25 expressing regulatory T-cells (FoxP3+ CD25+ CD4+ cells × 100 divided by the number of CD4+cells) (F); the percentage of FoxP3- T-cells (FoxP3-CD4+cells × 100 divided by the number of CD4+ cells) was decreased (G). The number of animals evaluated was n = 19 (sham) and n = 17 (STZ). Differences between the indicated cohorts are indicated as tendency, (B) [a]P = 0.057 or as significant, (E) [b]P = 0.018; (F) [c]P = 0.021; (G) [d]P = 0.034. STZ: Streptozotocin-treated.

Research conclusions

Our preclinical study on mice supports the idea that an aggressive adjustment of blood glucose concentration might not be necessary as a prerequisite for the treatment of patients with autoimmune pancreatitis.

Research perspectives

A clinical study that evaluates whether a tight adjustment of blood glucose is beneficial or harmful to patients should be pursued.

for excellent technical assistance.

REFERENCES

1　**SARLES H**, Sarles JC, Muratore R, Guien C. Chronic inflammatory sclerosis of the pancreas--an autonomous pancreatic disease? *Am J Dig Dis* 1961; **6**: 688-698 [PMID: 13746542 DOI: 10.1007/BF02232341]

2　**Kamisawa T**, Chari ST, Giday SA, Kim MH, Chung JB, Lee KT, Werner J, Bergmann F, Lerch MM, Mayerle J, Pickartz T, Lohr M, Schneider A, Frulloni L, Webster GJ, Reddy DN, Liao WC, Wang HP, Okazaki K, Shimosegawa T, Kloeppel G, Go VL. Clinical profile of autoimmune pancreatitis and its histological subtypes: an international multicenter survey. *Pancreas* 2011; **40**: 809-814 [PMID: 21747310 DOI: 10.1097/MPA.0b013e3182258a15]

3　**Shimosegawa T**, Chari ST, Frulloni L, Kamisawa T, Kawa S, Mino-Kenudson M, Kim MH, Klöppel G, Lerch MM, Löhr M, Notohara K, Okazaki K, Schneider A, Zhang L; International Association of Pancreatology. International consensus diagnostic criteria for autoimmune pancreatitis: guidelines of the International Association of Pancreatology. *Pancreas* 2011; **40**: 352-358 [PMID: 21412117 DOI: 10.1097/MPA.0b013e3182142fd2]

4　**Omiyale AO**. Autoimmune pancreatitis. *Gland Surg* 2016; **5**: 318-326 [PMID: 27294040 DOI: 10.21037/gs.2015.11.02]

5　**Kleeff J**, Welsch T, Esposito I, Löhr M, Singer R, Büchler MW, Friess H. Autoimmune pancreatitis--a surgical disease? *Chirurg* 2006; **77**: 154-165 [PMID: 16208510 DOI: 10.1007/s00104-005-1084-0]

6　**Ectors N**, Maillet B, Aerts R, Geboes K, Donner A, Borchard F, Lankisch P, Stolte M, Lüttges J, Kremer B, Klöppel G. Non-alcoholic duct destructive chronic pancreatitis. *Gut* 1997; **41**: 263-268 [PMID: 9301509 DOI: 10.1136/gut.41.2.263]

7　**Miyoshi H**, Uchida K, Taniguchi T, Yazumi S, Matsushita M, Takaoka M, Okazaki K. Circulating naïve and CD4+CD25high regulatory T cells in patients with autoimmune pancreatitis. *Pancreas* 2008; **36**: 133-140 [PMID: 18376303 DOI: 10.1097/MPA.0b013e3181577553]

8　**Hori S**, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; **299**: 1057-1061 [PMID: 12522256 DOI: 10.1126/science.1079490]

9　**Yagi H**, Nomura T, Nakamura K, Yamazaki S, Kitawaki T, Hori S, Maeda M, Onodera M, Uchiyama T, Fujii S, Sakaguchi S. Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. *Int Immunol* 2004; **16**: 1643-1656 [PMID: 15466453 DOI: 10.1093/intimm/dxh165]

10　**Fukumura Y**, Takase M, Mitani K, Suda K, Imamhasan A, Nobukawa B, Ueda A, Abe H, Yao T. Amount of CD4+CD25+ regulatory T cells in autoimmune pancreatitis and pilonidal sinus. *Pancreas* 2012; **41**: 910-915 [PMID: 22466163 DOI: 10.1097/MPA.0b013e31823e3c66]

11　**Ito T**, Nishimori I, Inoue N, Kawabe K, Gibo J, Arita Y, Okazaki K, Takayanagi R, Otsuki M. Treatment for autoimmune pancreatitis: consensus on the treatment for patients with autoimmune pancreatitis in Japan. *J Gastroenterol* 2007; **42** Suppl 18: 50-58 [PMID: 17520224 DOI: 10.1007/s00535-007-2051-y]

12　**Kamisawa T**, Egawa N, Inokuma S, Tsuruta K, Okamoto A, Kamata N, Nakamura T, Matsukawa M. Pancreatic endocrine and exocrine function and salivary gland function in autoimmune pancreatitis before and after steroid therapy. *Pancreas* 2003; **27**: 235-238 [PMID: 14508128 DOI: 10.1097/00006676-200310000-00007]

13　**Ito T**, Nakamura T, Fujimori N, Niina Y, Igarashi H, Oono T, Uchida M, Kawabe K, Takayanagi R, Nishimori I, Otsuki M, Shimosegawa T. Characteristics of pancreatic diabetes in patients with autoimmune pancreatitis. *J Dig Dis* 2011; **12**: 210-216 [PMID: 21615876 DOI: 10.1111/j.1751-2980.2011.00498.x]

14　**Miyamoto Y**, Kamisawa T, Tabata T, Hara S, Kuruma S, Chiba K, Inaba Y, Kuwata G, Fujiwara T, Egashira H, Koizumi K, Sekiya R, Fujiwara J, Arakawa T, Momma K, Asano T. Short and long-term outcomes of diabetes mellitus in patients with autoimmune pancreatitis after steroid therapy. *Gut Liver* 2012; **6**: 501-504 [PMID: 23170157 DOI: 10.5009/gnl.2012.6.4.501]

15　**Nishimori I**, Tamakoshi A, Kawa S, Tanaka S, Takeuchi K, Kamisawa T, Saisho H, Hirano K, Okamura K, Yanagawa N, Otsuki M; Research Committee on Intractable Pancreatic Diseases, the Ministry of Health and Welfare of Japan. Influence of steroid therapy on the course of diabetes mellitus in patients with autoimmune pancreatitis: findings from a nationwide survey in Japan. *Pancreas* 2006; **32**: 244-248 [PMID: 16628078 DOI: 10.1097/01.mpa.0000202950.02988.07]

16　**Angelopoulos N**, Dervenis C, Goula A, Rombopoulos G, Livadas S, Kaltsas D, Kaltzidou V, Tolis G. Endocrine pancreatic insufficiency in chronic pancreatitis. *Pancreatology* 2005; **5**: 122-131 [PMID: 15849483 DOI: 10.1159/000085264]

17　**Czakó L**, Hegyi P, Rakonczay Z Jr, Wittmann T, Otsuki M. Interactions between the endocrine and exocrine pancreas and their clinical relevance. *Pancreatology* 2009; **9**: 351-359 [PMID: 19454837 DOI: 10.1159/000181169]

18　**Zechner D**, Spitzner M, Bobrowski A, Knapp N, Kuhla A, Vollmar B. Diabetes aggravates acute pancreatitis and inhibits pancreas regeneration in mice. *Diabetologia* 2012; **55**: 1526-1534 [PMID: 22327285 DOI: 10.1007/s00125-012-2479-3]

19　**Zechner D**, Knapp N, Bobrowski A, Radecke T, Genz B, Vollmar B. Diabetes increases pancreatic fibrosis during chronic inflammation. *Exp Biol Med (Maywood)* 2014; **239**: 670-676 [PMID: 24719378 DOI: 10.1177/1535370214527890]

20　**Kanno H**, Nose M, Itoh J, Taniguchi Y, Kyogoku M. Spontaneous development of pancreatitis in the MRL/Mp strain of mice in autoimmune mechanism. *Clin Exp Immunol* 1992; **89**: 68-73 [PMID: 1352748 DOI: 10.1111/j.1365-2249.1992.tb06879.x]

21　**Fitzner B**, Holzhueter SA, Ibrahim S, Nizze H, Jaster R. Interferon-gamma treatment accelerates and aggravates autoimmune pancreatitis in the MRL/Mp-mouse. *Pancreatology* 2009; **9**: 233-239 [PMID: 19407476 DOI: 10.1159/000199434]

22　**Frossard JL**, Pastor CM. Experimental acute pancreatitis: new insights into the pathophysiology. *Front Biosci* 2002; **7**: d275-d287 [PMID: 11779694]

23　**Sah RP**, Dudeja V, Dawra RK, Saluja AK. Cerulein-induced chronic pancreatitis does not require intra-acinar activation of trypsinogen in mice. *Gastroenterology* 2013; **144**: 1076-1085.e2 [PMID: 23354015 DOI: 10.1053/j.gastro.2013.01.041]

24　**Schwaiger T**, van den Brandt C, Fitzner B, Zaatreh S, Kraatz F, Dummer A, Nizze H, Evert M, Bröker BM, Brunner-Weinzierl MC, Wartmann T, Salem T, Lerch MM, Jaster R, Mayerle J. Autoimmune pancreatitis in MRL/Mp mice is a T cell-mediated disease responsive to cyclosporine A and rapamycin treatment. *Gut* 2014; **63**: 494-505 [PMID: 23564336 DOI: 10.1136/gutjnl-2012-303635]

25　**Ehlers L**, Rohde S, Ibrahim S, Jaster R. Adoptive transfer of CD3+ T cells and CD4+ CD44 high memory T cells induces autoimmune pancreatitis in MRL/MpJ mice. *J Cell Mol Med* 2018; **22**: 2404-2412 [PMID: 29383850 DOI: 10.1111/jcmm.13537]

26　**Kaminitz A**, Mizrahi K, Askenasy N. Surge in regulatory T cells does not prevent onset of hyperglycemia in NOD mice: immune profiles do not correlate with disease severity. *Autoimmunity* 2014; **47**: 105-112 [PMID: 24328490 DOI: 10.3109/08916934.2013.866103]

27　**Zhen Y**, Sun L, Liu H, Duan K, Zeng C, Zhang L, Jin D, Peng J, Ding W, Zhao Y. Alterations of peripheral CD4+CD25+Foxp3+ T regulatory cells in mice with STZ-induced diabetes. *Cell Mol Immunol* 2012; **9**: 75-85 [PMID: 21983870 DOI: 10.1038/cmi.2011.37]

28　**Kamisawa T**, Okazaki K, Kawa S, Ito T, Inui K, Irie H, Nishino T, Notohara K, Nishimori I, Tanaka S, Nishiyama T, Suda K, Shiratori K, Tanaka M, Shimosegawa T; Working Committee of the Japan Pancreas Society and the Research Committee for Intractable Pancreatic Disease supported by the Ministry of Health, Labour and Welfare of Japan. Amendment of the Japanese Consensus Guidelines for Autoimmune Pancreatitis, 2013 III. Treatment and prognosis of autoimmune pancreatitis. *J Gastroenterol* 2014; **49**: 961-970 [PMID: 24639058 DOI: 10.1007/s00535-014-0945-z]

29　**Kamisawa T**, Shimosegawa T, Okazaki K, Nishino T, Watanabe H, Kanno A, Okumura F, Nishikawa T, Kobayashi K, Ichiya T, Takatori

H, Yamakita K, Kubota K, Hamano H, Okamura K, Hirano K, Ito T, Ko SB, Omata M. Standard steroid treatment for autoimmune pancreatitis. *Gut* 2009; **58**: 1504-1507 [PMID: 19398440 DOI: 10.1136/gut.2008.172908]

30   **Farris AB 3rd**, Lauwers GY, Deshpande V. Autoimmune pancreatitis-related diabetes: quantitative analysis of endocrine islet cells and inflammatory infiltrate. *Virchows Arch* 2010; **457**: 329-336 [PMID: 20632032 DOI: 10.1007/s00428-010-0948-y]

Basic Study

Moxibustion treatment modulates the gut microbiota and immune function in a dextran sulphate sodium-induced colitis rat model

Qin Qi, Ya-Nan Liu, Xiao-Ming Jin, Lin-Shuang Zhang, Cun Wang, Chun-Hui Bao, Hui-Rong Liu, Huan-Gan Wu, Xiao-Mei Wang

Qin Qi, Ya-Nan Liu, Cun Wang, Yueyang Clinical Medical College, Shanghai University of Traditional Chinese Medicine, Shanghai 200437, China

Xiao-Ming Jin, Stark Neurosciences Research Institute and Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, IN 46202, United States

Lin-Shuang Zhang, Zhejiang Institute for Food and Drug Control, Hangzhou 310052, Zhejiang Province, China

Chun-Hui Bao, Hui-Rong Liu, Huan-Gan Wu, Xiao-Mei Wang, Shanghai Research Institute of Acupuncture-moxibustion and Meridian, Shanghai University of Traditional Chinese Medicine, Shanghai 200030, China

ORCID number: Qin Qi (0000-0002-0563-1560); Ya-Nan Liu (0000-0002-3290-9206); Xiao-Ming Jin (0000-0002-8671-8640); Lin-Shuang Zhang (0000-0003-4043-8528); Cun Wang (0000-0003-4441-817X); Chun-Hui Bao (0000-0002-9763-0046); Hui-Rong Liu (0000-0002-9697-5085); Huan-Gan Wu (0000-0003-1725-6881); Xiao-Mei Wang (0000-0003-1962-0827).

Author contributions: Wang XM conceived and designed the study; Qi Q and Wang XM wrote the main manuscript text; Qi Q and Liu YN performed animal experiments; Jin XM collected data; Wang C and Bao CH analysed data; Liu HR and Wu HG prepared figures and tables; Jin XM and Zhang LS edited and improved the manuscript; all authors reviewed the manuscript.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (IACUC protocol number: 201611006).

Conflict-of-interest statement: To the best of our knowledge, no conflict of interest exists.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Manuscript source: Unsolicited manuscript

Correspondence to: Xiao-Mei Wang, MD, PhD, Associate Professor, Shanghai Research Institute of Acupuncture-moxibustion and Meridian, Shanghai University of Traditional Chinese Medicine, 650 South Wanping Road, Xuhui District, Shanghai 200030, China. wxm123@vip.sina.com
Telephone: +86-21-64381106

Abstract

AIM

To investigate the effect and mechanism of moxibustion in rats with ulcerative colitis.

METHODS

A rat colitis model was established by administering 4% dextran sulphate sodium solution. Seventy male rats were randomly divided into seven groups: Healthy controls (HC), ulcerative colitis model group (UC), UC with 7 d of moxibustion (UC-7), UC with 14 d of moxibustion (UC-14), UC with mesalazine gavage (UC-W), HC with 7 d of moxibustion (HC-7), HC with 14 d of moxibustion (HC-14). Moxibustion was applied to the bilateral Tianshu (ST25). Gut microbiome profiling was conducted by 16S rRNA amplicon sequencing, and PCR and ELISA determined the expression of inflammatory cytokines in colon mucosa and serum, respectively.

RESULTS

Moxibustion treatment restored the colonic mucosa and decreased submucosal inflammatory cell infiltration in colitis rats. Rats treated with moxibustion and mesalazine had significantly lower levels of the dominant phyla Proteobacteria and the genera *Saccharibacteria*, *Sphingomonas* and *Barnesiella* than colitis rats, and they could restore the microbiome to levels similar to those observed in healthy rats. UC rats had reduced alpha diversity, which could be alleviated by moxibustion therapy, and UC-7 had a higher alpha diversity than UC-14. This finding suggests that short-term (7 d) but no longer term (14 d) moxibustion treatment may significantly affect the gut microbiome. The potential bacterial functions affected by moxibustion may be ascorbate and aldarate metabolism, and amino acid metabolism. Compared with HC group, the levels of the cytokines interleukin-12 (IL-12) ($P < 0.05$) and IL-6, IL-17, IL-23, interferon-γ, lipopolysaccharide, IgA, tumour necrosis factor-α and its receptors 1 (TNFR1) and TNFR2 ($P < 0.01$) were all increased, whereas anti-inflammatory cytokine IL-2 and IL-10 ($P < 0.01$) and transforming growth factor-β ($P < 0.05$) were decreased in UC rats. These changes were reversed by moxibustion.

CONCLUSION

Our findings suggest that moxibustion exerts its therapeutic effect by repairing mucosal tissue damage and modulating the gut microbiome and intestinal mucosal immunity.

Key words: Ulcerative colitis; Moxibustion; 16S rRNA; Gut microbiome; Inflammatory cytokine

Core tip: Ulcerative colitis (UC) is a form of inflammatory bowel disease and microbial dysbiosis is an important pathological factor of UC. Moxibustion treatment can repair mucosal tissue damage and regulate immune function in patients with UC associated with gut microbiome changes.

In this study, moxibustion can significantly reduce the colonic tissue damage and the levels of pro-inflammatory cytokines and anti-inflammatory cytokines in rats with 4% dextran sulphate sodium-induced ulcerative colitis, relieve intestinal inflammation, and promote the restoration of impaired colonic tissue. In addition, moxibustion can increase the diversity of the gut microbiome and the UC with 7 d of moxibustion had a higher alpha diversity than UC with 14 d of moxibustion.

INTRODUCTION

The gut microbiome has been referred to as a "forgotten organ"[1] due to its capacity to influence host physiology. The gut microbiome is incredibly diverse, with dominant phyla including Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, Actinobacteria and Fusobacteria[2,3]. Gut microbiome composition varies substantially among individuals and is thought to be a key determinant of susceptibility to several diseases[4-6]. The gut microbiome provides signals that promote immune cell maturation and ensure the normal development of immune function[7], which is crucial for human life and profoundly influences human physiology and metabolism[8,9].

Ulcerative colitis (UC) is a form of inflammatory bowel disease that can lead to bloody diarrhoea, passage of pus and/or mucus, and abdominal cramping during bowel movements[10,11]. Immune dysfunction plays a key role in driving inflammatory responses during UC development and progression. UC is commonly treated with therapeutics such as the anti-inflammatory drugs mesalazine and sulphasalazine, with steroid enemas[12] or with surgical intervention. The aetiology and pathogenesis of UC have not been fully elucidated but are thought to result from detrimental interactions among intestinal microbiota, the gut epithelium, and the host immune system in genetically susceptible individuals[13,14]. Several studies have recently reported decreased gut microbial diversity in patients with active UC[15,16]. Microbial dysbiosis is an important pathological factor of UC that affects host mucosal immunity[17-19]. Pathogenic bacteria and opportunistic pathogens interact with the intestinal mucosa directly or indirectly *via* secreted toxins, which may lead to an inappropriate intestinal mucosal immune response.

Studies have demonstrated that moxibustion, a traditional Chinese therapy that treats and prevents diseases using moxa (floss of *Artemisia argyi*), is a safe and effective treatment for UC[20-23]. Moxibustion

exerts its therapeutic effects by regulating immune function[24,25], apoptosis[26,27], and protein expression at acupuncture points and in colon tissues[28,29]. UC is associated with changes in the gut microbiome, and moxibustion modulates the imbalance of intestinal flora[30]. Nevertheless, the gut microbiome is a complex community with only a small proportion of culturable organisms, and the effect of moxibustion on the gut microbiome remains unknown. Recently developed culture-independent high-throughput sequencing methods allow gut microbiome profiling and assessments of microbial gene abundance and community structure to be performed more easily[31].

Here, we used 16S rRNA gene sequencing to assess the gut microbial composition and community structure in faecal samples of UC rats treated with moxibustion or mesalazine gavage. Our aims were to determine the impact of moxibustion on intestinal microbial community structure, the role of the intestinal microbiota in UC pathogenesis, and the molecular mechanism underlying the effect of moxibustion in UC treatment.

MATERIALS AND METHODS

Rats and groups

Seventy specific pathogen-free male Sprague Dawley rats (body weight 180-220 g) were provided by the Department of Laboratory Animal Science of Fudan University (Shanghai Slack Laboratory Animal Co., LTD. license: SCXK (Shanghai) 2012-0002 and SYXK (Shanghai) 2009-0082). All rats were raised under standard conditions: $25 \pm 1\,℃$, 50%-70% humidity, and 12 h light/dark cycle. Rats were allowed to acclimatize for one week before the start of the trial. Rats ($n = 70$) were divided into seven groups: healthy controls (HC), healthy controls with seven days of moxibustion (HC-7), healthy controls with fourteen days of moxibustion (HC-14), UC model group (UC), UC model with seven days of moxibustion (UC-7), UC model with fourteen days of moxibustion (UC-14), and UC model with mesalazine gavage (UC-W). UC was induced with 4% dextran sulphate sodium (DSS; molecular weight: 36000-50000 Da; Art.No.9011-18-1; MP Biomedicals) supplied in drinking water. Animal breeding, care and all experiments were performed according to procedures approved by the University Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (IACUC protocol number: SYXK (shanghai) 2009-0082) to relieve pain and avoid injury. All protocols were performed in strict accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, as formulated by the Ministry of Science and Technology of the People's Republic of China[32]. We tried all efforts to minimize animal suffering.

DSS-induced-colitis

UC was induced with 4% DSS in drinking water for seven consecutive days as described previously[33]. After seven days, two rats were randomly selected to confirm successful establishment of the animal model. Rats were provided 1% DSS in the drinking water for seven consecutive days in the UC, UC-7 and UC-W groups and fourteen consecutive days in the UC-14 group.

Treatment

For the HC-7 ($n = 10$) and UC-7 ($n = 10$) groups, moxa cones (0.5 cm in diameter and 0.6 cm high) (Nanyang Hanyi Moxa Co., Ltd. China) were placed on the herb cake, and the herb cake was placed on Tianshu (ST25, bilateral) with moxibustion for 10 min once a day for seven days, the herb cakes were prepared with Chinese medicine powder and yellow wine into size of 1.0 cm in diameter and 0.5 cm in thickness, and the skin temperature of acupuncture points was $43 \pm 1\,℃$. ST25 is 5 mm lateral to anterior midline at the navel level[34]. The HC-14 ($n = 10$) and UC-14 ($n = 10$) groups received the same treatment as the HC-7 and UC-7 groups, respectively, but for fourteen days. The UC-W group ($n = 10$) received mesalazine enteric-coated tablets (Heilongjiang Tianhong Pharmaceutical Co., Ltd; Lot number: H20103359) by gavage administration twice daily for seven consecutive days with a dose calculated from that specified for an adult of 70 kg and a weight ratio of 1:0.018 for rats (200 g). The HC ($n = 10$) and UC ($n = 10$) groups did not receive any treatment but received the same fixation (to fix the hands and feet on the fixed mount).

Sample collection and processing

Following treatment, rats were anaesthetized *via* intraperitoneal injection with 2% pentobarbital sodium for tissue collection. Blood samples were collected by abdominal aortic, after one hour standing, the samples were centrifuged at 3000 rpm for ten minutes at $4\,℃$ to separate the plasma. The obtained plasma was stored at $-80\,℃$. Colon samples were rapidly removed and separated from the large intestine (6 cm distal to the anus). Then, 5 g of faecal matter was collected in cryogenic vials and stored at $-80\,℃$. Colon samples were flushed with physiological saline to remove the surrounding connective tissue and fat. Colonic mucosal injury was observed with the naked eye. Then, the colon was divided into two segments: one segment was fixed in 10% paraformaldehyde for haematoxylin and eosin (HE) staining for histological observation and the histological grade was scored with the bland method[30] (Table 1), whereas the other segment was stored at $-80\,℃$.

Haematoxylin and eosin staining

Colon samples fixed with 10% paraformaldehyde were dehydrated and immersed in wax using an automatic tissue dehydrating machine, embedded in paraffin and sectioned at 4 μm thickness with a microtome. Slices were incubated with xylene Ⅰ and Ⅱ for 20 min each and rehydrated in absolute alcohol, 95% alcohol, 85% alcohol and 75% alcohol (3 min each time). Slices were stained with haematoxylin for 1 min, followed by 1% hydrochloric acid-alcohol for 30 s, and counterstained

Table 1 Histopathological Scores

Histopathological manifestation		score
Ulcer	No ulcer	0
	Ulcer area < 3 cm	1
	Ulcer area > 3 cm	2
Inflammation	No inflammation	0
	Mild inflammation	1
	Severe inflammation	2
Granuloma	No granuloma	0
	Granuloma	1
Lesion depth	No lesion	0
	Submucosa	1
	Muscular layer	2
	Serosa layer	3
Fibrosis	No fibrosis	0
	Mild fibrosis	1
	Severe fibrosis	2

with eosin for 5 min. Sample histology was observed by electron microscope.

Bacterial genomic DNA extraction

Genomic DNA was extracted from faecal samples using the QIAamp DNA Mini Kit (QIAGEN, Germany). Briefly, 20 μL of proteinase K solution (20 mg/mL) and 100 mg of zirconium beads (0.1 mm) were added to the pellet. The mixture was agitated three times with a Mini-Beadbeater (FastPrep, Thermo Electron Corporation, United States); buffer AL was added to the mixture, which was then incubated for 10 min at 70 ℃. Next, 200 μL of ethanol (96%) was added. This mixture was then loaded onto a QIAamp Mini spin column and centrifuged at 8000 *g* for 1 min. The column material was washed with the first washing buffer (buffer AW1, 500 μL) followed by the second washing buffer (buffer AW2, 500 μL) provided with the kit. Finally, DNA was eluted with 100 μL of buffer AE. DNA integrity was assessed by electrophoresis on a 1% agarose gel containing 0.5 mg/mL ethidium bromide. DNA concentrations were determined using a NanoDrop ND-2000 spectrophotometer (Thermo Electron Corporation).

Illumina sequencing and data processing

The 16S rRNA gene hypervariable V3-V4 region was amplified by PCR using the barcode-tag universal bacterial modified primers. The prototype primers were 341F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACVVGGGTATCTAATC-3'). Amplicon sequencing was performed using Illumina MiSeq (PE300). Raw reads were demultiplexed and filtered according to read length and quality. Chimeric reads were removed, and OTU selection was performed as previously described[35]. The filtered reads were dereplicated to unique sequences and sorted by abundance before being subjected to OTU clustering at 97% similarity. Taxonomy was assigned to the representative sequence of each OTU using the Ribosomal Database Project classifier[36].

Prediction of metagenome functional content

Phylogenetic Investigation of Communities by Reconstruc-

tion of Unobserved States (PICRUSt 1.0.0)[37] was used to predict metagenome function from the 16S rRNA data. Statistical Analysis of Metagenomic Profiles (STAMP)[38] was used to analyse the predicted metagenome and to identify pathways associated with UC. The Benjamini-Hochberg procedure was used to control the false discovery rate due to multiple testing.

PCR analysis

Total RNA was isolated using TRIzol® LS (Invitrogen Corp.) according to the manufacturer's instructions. The integrity of the RNA samples was assessed *via* agarose gel (10 g/L) electrophoresis, and RNA concentrations were determined using a NanoDrop ND-2000 Spectrophotometer (Thermo Electron Corporation). Two micrograms of total RNA was used for RT-PCR in a 20 μL reverse transcription reaction system. Two micrograms of RNA, up to 8 μL of DEPC, 0.5 μL (50 U/μL) of RNase inhibitor and 2 μL of oligomer (dT)15 and primer were mixed and placed in a 65 ℃ water for 5 min. Then, the mixture was placed at room temperature for 10 min. After a 5 s centrifugation, the following reagents were added to the reactions: 0.5 μL (50 U/μL) of RNase inhibitor, 4 μL of 5 × RT buffer solution, 2 μL of dNTPs (10 mmol/L for each), 2 μL of DTT, and 1 μL of AMV reverse transcriptase (200 U/μL). The solution was mixed and placed in water at 40 ℃ for 1 h, heated to 90 ℃ in water for 5 min, and centrifuged for 5 s.

The primers used were designed with Primer Express Software v2.0 (Applied Biosystems, United States) and synthesized at the Beijing Genomics Institute. Gene sequences of the primers are presented in Table 2.

Each 16 μL PCR included: 8 μL of 2 × SYBR Green PCR mix, 1 μL of each sense and antisense primer, 1 μL of reverse transcription product (cDNA), and 5 μL of H_2O. RT-PCR was performed using a ViiA 7 Real Time PCR System (Applied Biosystems, United States). The reaction conditions were set at 95 ℃ for 2 min, 94 ℃ for 10 s, 60 ℃ for 10 s, and 72 ℃ for 40 s for 40 cycles.

ELISA analysis

The levels of interleukin-2, 10, 12, 17, 23, interferon-γ (IFN-γ), lipopolysaccharide (LPS), immunoglobulin A (IgA), transforming growth factor-β (TGF-β), tumour necrosis factor-α (TNF-α), TNF receptor 1 (TNFR1) and TNFR2 in all serum samples were tested by sandwich ELISA. According to manufacturer's instructions, IL-2, IL-10, IL-12, 17, IL-23, IFN-γ, LPS, IgA, TGF-β, TNF-α, TNFR1 and TNFR2 were measured using corresponding ELISA kits (Biotech well, Shanghai, China). The detection limits were: 17 pg/mL for IL-2, IL-12, IL-23, and TNF-α, 16 pg/mL for IL-10, 15 pg/mL for IL-17, and TGF-β, 12 pg/mL for IFN-γ, 8 pg/mL for LPS, 8ng/mL for IgA, 6 pg/mL for TNFR1, and 5 pg/mL for TNFR2.

Statistical analysis

Differential abundance testing between groups was performed using the Kruskal-Wallis test in R 3.2.3 (http://cran.r-project.org). Alpha diversity was measured with

Table 2 Primer sequences		
Target gene	**Forward sequence**	**Reverse sequence**
IL-6	GCCAGAGTCATTCAGAGCAA	TTGGTCCTTAGCCACTCCTT
IL-10	CTGTCATCGATTTCTCCCCT	CAAACTCATTCATGGCCTTG
IL-12	CCCTCAAGTTCTTCGTCCGC	TTTGCATTGGACTTCGGCAG
IL-17	ATCCAGCAAGAGATCCTGGT	CAATAGAGGAAACGCAGGTG
IL-23	TGTGGTGATGGTTGTGATCC	TGTGAAGATGTCCGAGTCCA
TNF-α	GAAAGCATGATCCGAGATGT	CAGGAATGAGAAGAGGCTGA
TNFR1	TGAGACGCATTTCCAGTGTG	AGAATCCTGCGTGGCAGTTA
TNFR2	CATGTCAACGTCACCTGCAT	TCATCCTTTGGAGACCCTGA
rACTIN	ACTTCGAGCAGGAGATGGCC	CCCAGGAAGGAAGGCTGGAA

Figure 1 Body weight gains after treatment in different groups. HC: Healthy controls; UC: UC model group; UC-7: UC model with seven days of moxibustion; UC-14: UC model with fourteen days of moxibustion; UC-W: UC model with mesalazine gavage; HC-7: Healthy controls with seven days of moxibustion; HC-14: healthy controls with fourteen days of moxibustion. The body weight gain in the UC group was decreased compared with the HC group ($P < 0.01$). After treatment, the body weight gains in the UC-7, UC-14, HC-7, HC-14 ($P < 0.01$) and UC-W ($P < 0.05$) groups were increased compared with the UC group. [b]$P < 0.01$ *vs* the HC group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* the UC group.

Simpson and Shannon diversity indices. Beta diversity was determined using unweighted unifrac distance and was summarized by PCoA. A principal components analysis (PCA) plot of Kyoto Encyclopaedia of Genes and Genomes (KEGG) modules with significant differences in mean proportion differences was created using STAMP[39] (v2.1.3). Body weight gain, RT-PCR and ELISA analysis data are presented as the mean ± SD. Histopathological score data are expressed as M (Q25-Q75). ANOVA and Least Significant Difference multiple comparison tests were performed using IBM SPSS statistics 21.0 (IBM Co., Armonk, NY, United States). $P < 0.05$ was considered significant.

RESULTS

Body weight gains in different groups
Two rats in the UC-W group died after gavage during the experiment. The body weight gain in the UC group was lower than that in the HC group ($P < 0.01$). After treatment, the body weight gains in the UC-7, UC-14, HC-7, HC-14 ($P < 0.01$) and UC-W ($P < 0.05$) groups were higher than that in the UC group (Figure 1).

Changes in the colon histopathology
Rat colons were observed by light microscopy (Figure 2). The HC, HC-7, and HC-14 groups had similar histopathologies, with intact colonic mucosal epithelia, regularly arranged glands, and evenly distributed mesenchyme. No congestion, oedema, ulcers or other pathological abnormalities were noted. By contrast, UC rats exhibited colonic mucosa damage, with a reduced number of glands and substantial inflammatory cell infiltration in the mucosa and submucosa. These abnormalities could be alleviated by moxibustion treatment, which restored the colonic mucosa to the level of healthy rats, and decreased submucosal inflammatory cell infiltration and congestion in the UC-7 and UC-14 groups. However, in the UC-W group, mucosal and submucosal glands were disorganized, with abundant inflammatory cell infiltration. The histopathological scores for the colon tissues in the different groups are presented in Figure 3. The histopathological scores in the UC group were significantly higher than those in the HC group ($P < 0.01$). After treatment, the scores were decreased in the UC-7, UC-14, UC-W, HC-7 and HC-14 groups compared with those in the UC group ($P < 0.01$).

Core bacteria
Approximately 3.9 million high-quality (> 220 bp) sequence reads were obtained using the MiSeq system (Illumina, San Diego, CA, United States), which led to the identification of 1545 operational taxonomic units (OTUs), with an average read length of 451.58 bp. Overall, the
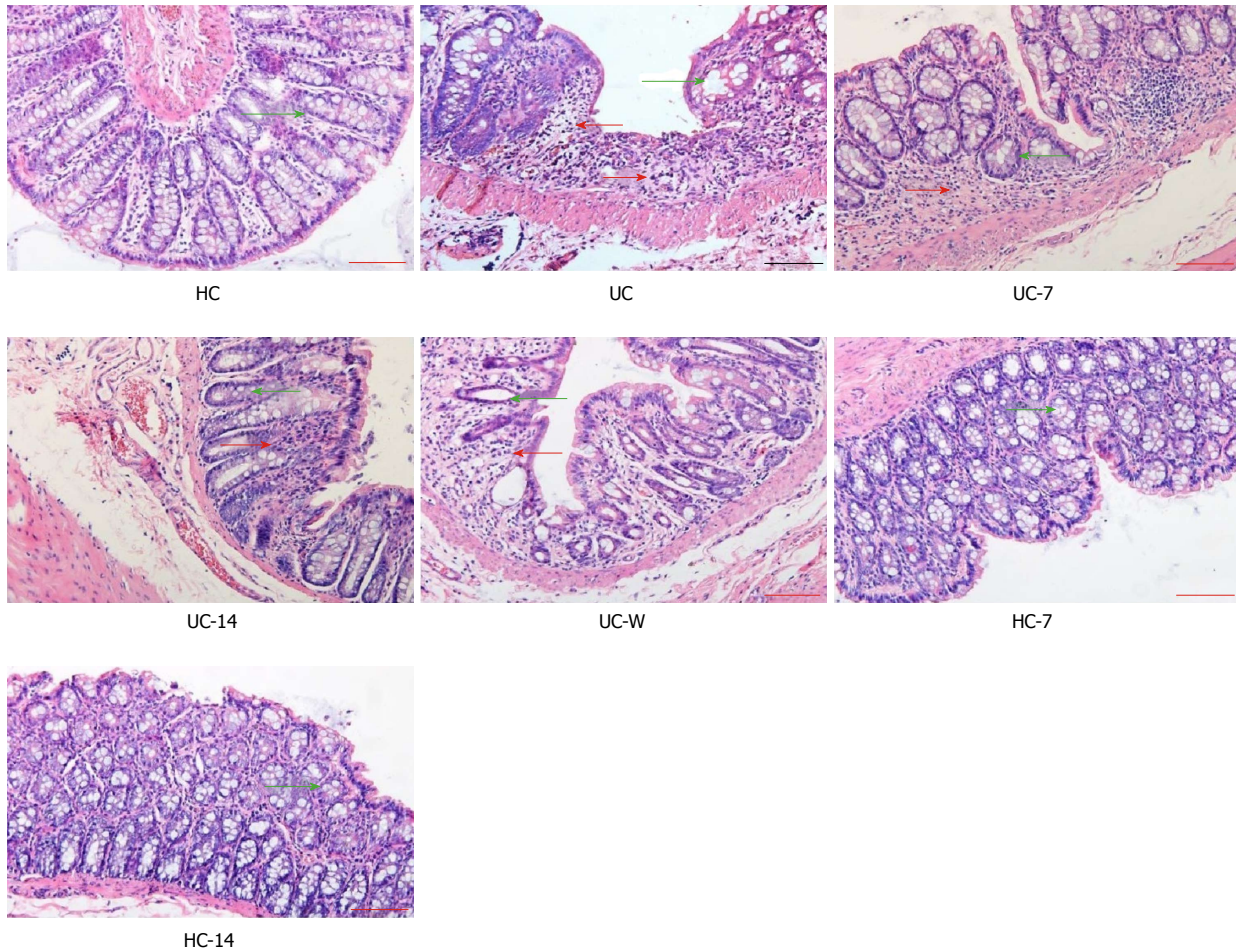
Figure 2 Rat colon tissue pathology under different treatments. HC: Healthy controls; UC: UC model group; UC-7: UC model with seven days of moxibustion; UC-14: UC model with fourteen days of moxibustion; UC-W: UC model with mesalazine gavage; HC-7: Healthy controls with seven days of moxibustion; HC-14: Healthy controls with fourteen days of moxibustion. The colonic mucosa in HC, HC-7, and HC-14 rats exhibited no pathological abnormalities. The colonic mucosa was damaged in the UC group, with a reduction in the number of glands and cell infiltration. After moxibustion treatment, decreased cell infiltration and congestion were noted in the UC-7 and UC-14 groups. Glands were disorganized with abundant inflammatory cell infiltration in the UC-W group. Red arrow indicated inflammatory cell infiltration and green arrow indicate gland (magnification: 200 ×).
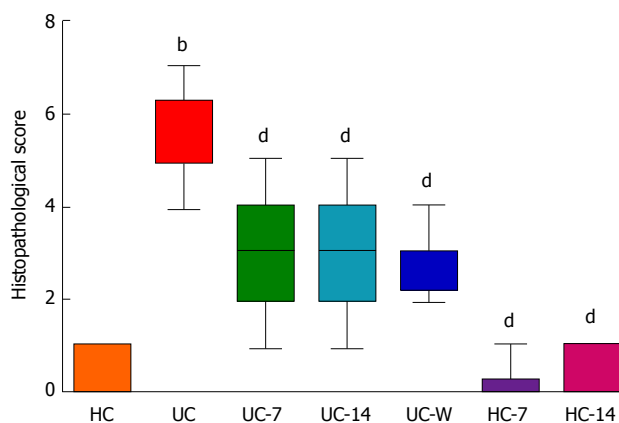

Figure 3 Histopathological scores for colon tissue in the different groups. HC: Healthy controls; UC: UC model group; UC-7: UC model with seven days of moxibustion; UC-14: UC model with fourteen days of moxibustion; UC-W: UC model with mesalazine gavage; HC-7: Healthy controls with seven days of moxibustion; HC-14: Healthy controls with fourteen days of moxibustion. The histopathological scores in the UC group were significantly increased compared with the HC group ($P < 0.01$). After treatment, the scores were decreased in the UC-7, UC-14, UC-W, HC-7 and HC-14 groups compared with the UC group ($P < 0.01$). [b]$P < 0.01$ *vs* the HC group; [d]$P < 0.01$ *vs* the UC group.

relative abundance levels of 18 dominant phyla were identified in the entire model system. Among these phyla, five (Firmicutes, Bacteroidetes, Proteobacteria, Candidatus Saccharibacteria, and Actinobacteria) were present at ≥ 1% and were the dominant phyla in all groups, but relative abundance varied by treatment. A total of 186 families were detected, and 100 families were commonly present, of which *Lachnospiraceae*, *Ruminococcaceae*, *Porphyromonadaceae*, *Lactobacillaceae*, *Sphingomonadaceae*, *Veillonellaceae* and *Coriobacteriaceae* were the dominant families detected. A total of 325 OTUs were identified at the genus level, of which 10 core OTUs were present in all groups. The highest average number of OTUs identified at the genus level (134) was detected in the UC-14 rats, whereas the lowest (78) was found in HC rats. As shown in Figure 4, the UC group had the most unique genera, even after treatment with either moxibustion or mesalazine gavage.

Community abundance variation between healthy controls and UC model rats

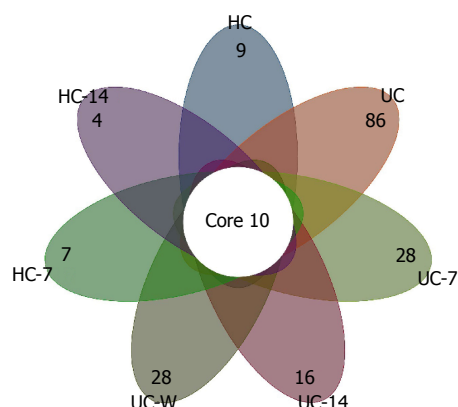There were significant differences in the microbiome

Figure 4 Summary of operational taxonomic units identified at the genus level in each group. HC: Healthy controls; UC: UC model group; UC-7: UC model with seven days of moxibustion; UC-14: UC model with fourteen days of moxibustion; UC-W: UC model with mesalazine gavage; HC-7: Healthy controls with seven days of moxibustion; HC-14: Healthy controls with fourteen days of moxibustion. There were ten core operational taxonomic units in all groups. The UC group had the most unique genera.

between healthy controls and UC rats. Although Firmicutes was the predominant phylum in all samples, UC rats had increased relative abundance levels of Proteobacteria, Candidatus and Saccharibacteria and decreased relative abundance levels of Firmicutes and Actinobacteria compared with HC rats. At the family level, UC rats had decreased *Lactobacillaceae* and *Ruminococcaceae* and increased *Sphingomonadaceae*, *Pseudomonadaceae* and *Porphyromonadaceae* (Figure 5B). In addition, *Lactobacillus*, *Clostridium* ⅩⅣ*a* and *Ruminococcus* were increased and *Saccharibacteria* and *Sphingomonas* were decreased in HC compared with UC rats (Figure 5C). UC rats had lower alpha diversity than HC rats, and the Shannon Diversity Index was reduced (Figure 5D), consistent with previous reports[15,40].

Community abundance variations between healthy controls with or without moxibustion treatment

In HC-7 and HC-14 groups, Firmicutes were decreased and Bacteroidetes and Proteobacteria were increased compared with the HC group (Figure 5A). At the family level, *Porphyromonadaceae* was increased and *Ruminococcaceae* and *Veillonellaceae* were decreased in moxibustion-treated groups (Figure 5B). Compared with the HC-7 group, the HC-14 group had increased abundance levels of *Lachnospiraceae* and *Ruminococcaceae* and a decreased abundance of *Lactobacillaceae*. At the genus level, *Lactobacillus* and *Desulfovibrio* were significantly increased, and *Ruminococcus* and *Barnesiella* were significantly decreased in both HC-7 and HC-14 groups relative to HCs. In particular, significant differences between HC-7 and HC-14 were noted regarding the abundance levels of *Lactobacillus*, *Saccharibacteria*, and *Blautia*. HC-7 exhibited increased abundance levels of *Lactobacillus* and *Blautia* and a reduced abundance of *Saccharibacteria* compared with those in the HC-14 group. *Lactobacillus*

and *Pseudomonas* were significantly enriched in HC-7 (but not HC-14) compared with the HC group. Interestingly, *Saccharibacteria* was decreased in HC-7 but increased in HC-14 compared with HCs (Figure 5C). It is not clear why the alpha diversity was decreased in HC-7 but increased in HC-14 compared with HCs (Figure 5D).

Community abundance variations between UC groups with or without moxibustion treatment

Moxibustion treatment (but not mesalazine gavage) for seven days or fourteen days was associated with an increase in Firmicutes compared with the UC group, whereas Proteobacteria and Candidatus and Saccharibacteria were decreased to varying degrees in both the moxibustion- and mesalazine-treated groups compared with the UC group. Bacteroidetes was reduced in UC-14 but substantially increased in the UC-W group compared with the UC group (Figure 5A). At the family level, UC-7 was similar to UC-W but significantly different from UC-14. UC-14 exhibited increased *Sphingomonadaceae* and decreased *Porphyromonadaceae* compared with UC-7 and UC-W. *Coriobacteriaceae*, *Pseudomonadaceae* and *Bifidobacteriaceae* were significantly reduced in UC-7, UC-14 and UC-W groups compared with the UC group (Figure 5B). UC-14 had relatively low levels of *Lactobacillus* but relatively high levels of *Clostridium XIVa* and *Sphingomonas* with a dominant position in all groups. In the UC-W group, *Saccharibacteria* was decreased and *Barnesiella*, *Prevotella,* and *Alloprevotella* were increased significantly compared with all of the other groups (Figure 5C).

Biodiversity and microbial richness

Alpha diversity was estimated through rarefaction analysis and the Shannon and Chao1 indexes. The lowest OTU richness was observed in the HC groups. Multiple rarefaction curves analysis indicated variation among samples and exhibited sufficient data coverage for diversity in the samples. The Shannon Wiener index showed the lowest value of evenness in HC rats. Surprisingly, HC-7 also showed a lower value. Interestingly, after seven days of treatment with moxibustion, the genus level diversity was comparable to that of HCs but notably different from the UC-14 and UC-W groups. UC-7 and UC-W had the highest median alpha diversity, which was restored to the normal level of HC and HC-14 (Figure 5D). This finding suggests that both moxibustion treatment and mesalazine gavage increased colonic microbial diversity. In terms of beta-diversity, UC samples clustered separately from HC samples in a principal coordinates analysis (PCoA), indicating that disease state was the primary factor influencing community differences (Figure 6). UC -7 was more similar to UC-W on the first axis, whereas HC-7 and HC-14 were more similar to HC and UC-14. Using non-metric multidimensional scaling (nMDS) instead of PCoA produced similar results (Figure 7).
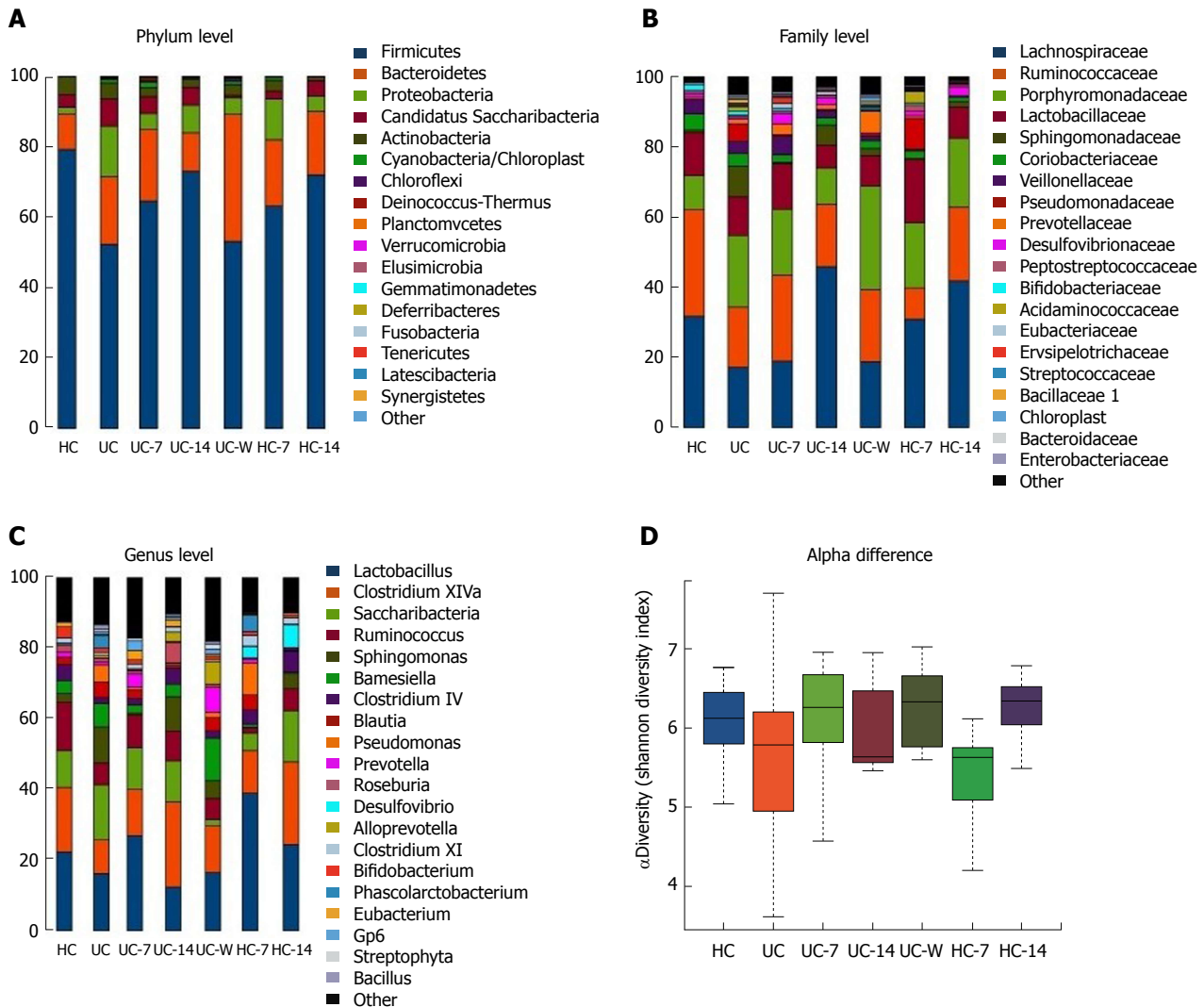
A

B

C

D

Figure 5 Comparative analyses of the dominant phyla, families and genera under different treatments. HC: Healthy controls; UC: UC model group; UC-7: UC model with seven days of moxibustion; UC-14: UC model with fourteen days of moxibustion; UC-W: UC model with mesalazine gavage; HC-7: Healthy controls with seven days of moxibustion; HC-14: Healthy controls with fourteen days of moxibustion. A: The relative abundance levels of Proteobacteria, *Candidatus*, and Saccharibacteria were increased, whereas Firmicutes and Actinobacteria were decreased in the UC group compared with the HC group. These changes in microbiota profiles could be reversed by moxibustion treatment for seven days or fourteen days; B: UC rats had decreased *Lactobacillaceae* and *Ruminococcaceae* and increased *Sphingomonadaceae*, *Pseudomonadaceae*, and *Porphyromonadaceae* compared with the HC group. After treatment, *Pseudomonadaceae* was reduced in the UC-7, UC-14 and UC-W groups compared with the UC group; C: *Lactobacillus*, *Clostridium* XIVa, and *Ruminococcus* were increased and Saccharibacteria and *Sphingomonas* were decreased in HC rats compared with UC rats; D: UC rats had decreased alpha diversity compared with HC rats. After treatment, alpha diversity was increased in the UC-7 and UC-W groups.

Genus level differences among treatment groups

To establish which taxa were contributing most to the apparent differences in diversity between groups, genus level abundance was assessed. *Saccharibacteria*, *Ruminococcus*, *Sphingomonas*, *Pseudomonas*, *Clostridium* Ⅳ, *Prevotella* and *Alloprevotella* were the most affected genera (Figure 8). These opportunistic pathogens can cause infection and may be important contributing factors in UC pathogenesis. Indeed, both Bacteroidetes[41] and *Clostridium* Ⅳ[42] are associated with UC. In our study, we found that UC rats had a higher density of Bacteroidetes and reduced *Clostridium* Ⅳ compared with the control.

Predictive functional profiling of microbial communities

Community function was inferred from 16S compositional data using PICRUSt. In the KEGG pathway

analysis, ascorbate and aldarate metabolism were significantly increased in both UC and HC rats treated with moxibustion for fourteen days (Supplement Table 1), which suggests that long-term moxibustion treatment could increase ascorbate and aldarate metabolism. Furthermore, compared with the HC group, the HC-7 and HC-14 groups had significant increases in genes belonging to the amino acid metabolism pathway. Compared with the UC group, these genes were increased in the UC-7 and UC-14 groups, but no significant differences were noted. However, the roles of these metabolic pathways in UC are unclear.

Levels of IL-6, IL-10, IL-12, IL-17, IL-23, TNF-α, TNFR1 and TNFR2 mRNA in colon mucosa

IL-6, IL-17, IL-23, TNF-α, TNFR1 and TNFR2 ($P <$ 0.01) and IL-12 mRNA ($P <$ 0.05) exhibited significant
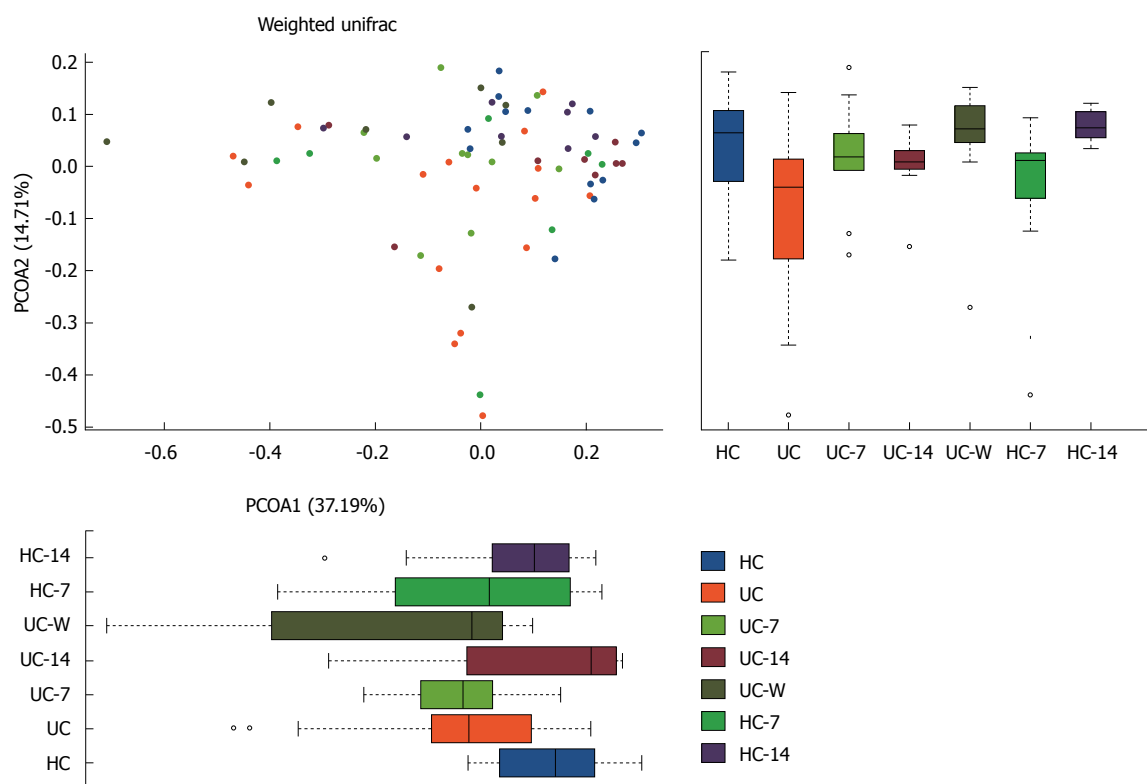
Figure 6　Beta diversity analyses by PCoA. HC: Healthy controls; UC: UC Model group; UC-7: UC model with seven days of moxibustion; UC-14: UC model with fourteen days of moxibustion; UC-W: UC model with mesalazine gavage; HC-7: Healthy controls with seven days of moxibustion; HC-14: Healthy controls with fourteen days of moxibustion. UC samples clustered separately from HC samples. The UC-7 group was more similar to the UC-W group on the first axis, whereas the HC-7 and HC-14 groups were more similar to the HC and UC-14 groups. Sample colour is based on treatment group.
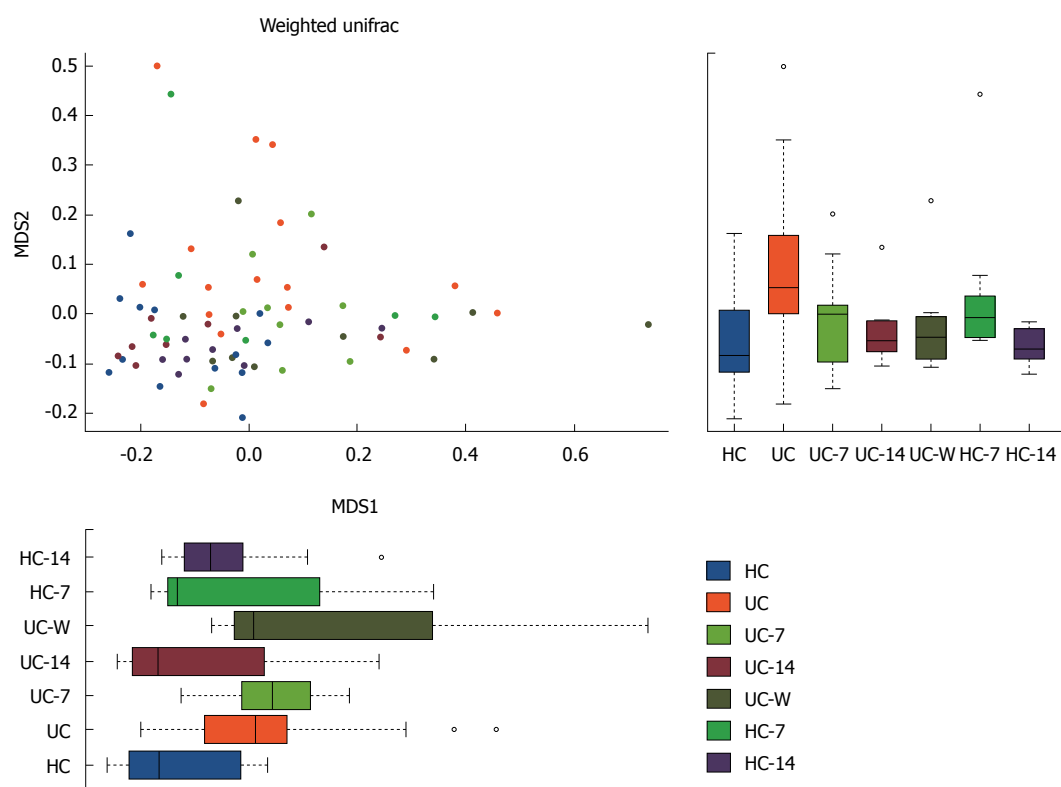

Figure 7　Non-metric multidimensional scaling between groups. HC: Healthy controls; UC: UC model group; UC-7: UC model with seven days of moxibustion; UC-14: UC model with fourteen days of moxibustion; UC-W: UC model with mesalazine gavage; HC-7: Healthy controls with seven days of moxibustion; HC-14: Healthy controls with fourteen days of moxibustion. UC samples clustered separately from HC samples. UC -7 was more similar to UC-W on the first axis. Sample colour is based on treatment group.
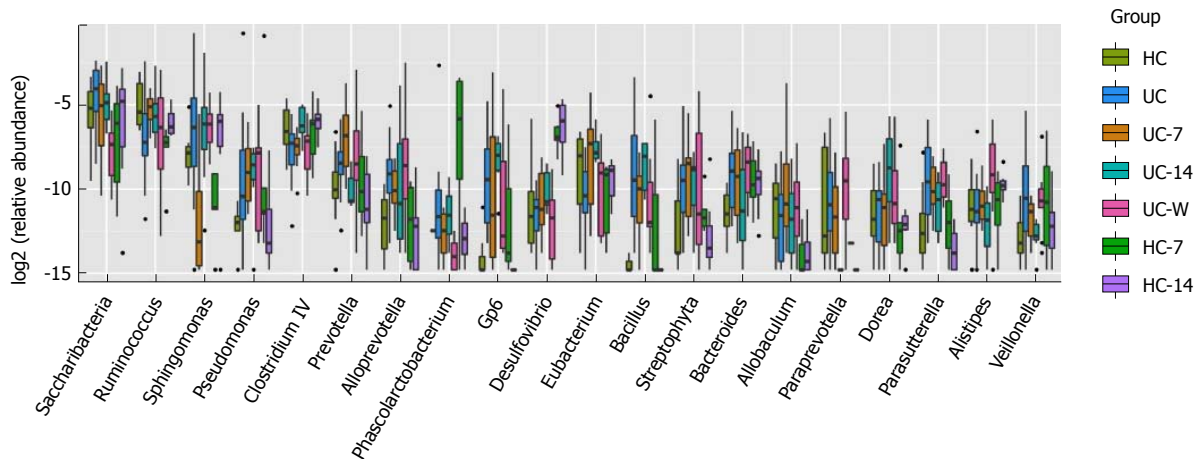
Figure 8 Genus-level differences between treatment groups. HC: Healthy controls; UC: UC model group; UC-7: UC model with seven days of moxibustion; UC-14: UC model with fourteen days of moxibustion; UC-W: UC model with mesalazine gavage; HC-7: Healthy controls with seven days of moxibustion; HC-14: Healthy controls with fourteen days of moxibustion. UC rats had a higher density of Bacteroidetes and a lower *Clostridium* IV density compared with HC rats.
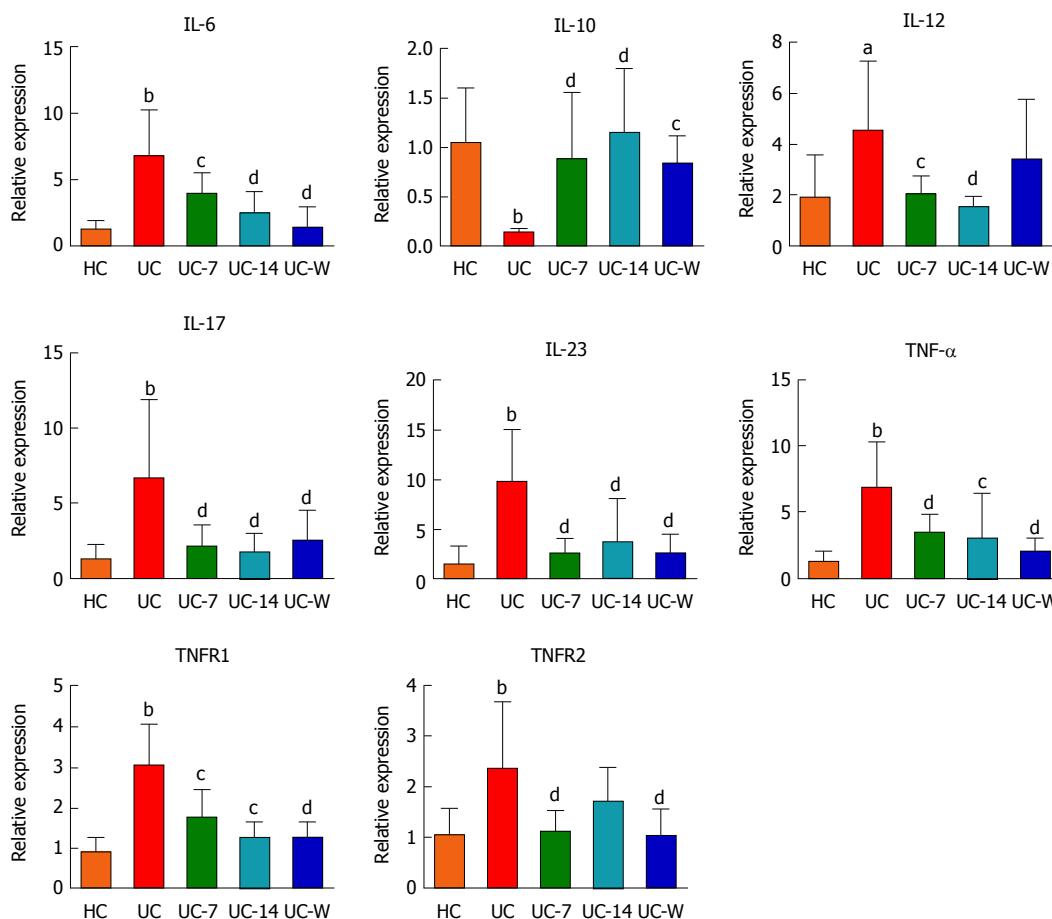

Figure 9 The relative expression levels of IL-6, IL-10, IL-12, IL-17, IL-23, TNF-α, TNFR1 and TNFR2 in colon mucosa. HC: Healthy controls; UC: UC model group; UC-7: UC model with seven days of moxibustion; UC-14: UC model with fourteen days of moxibustion; UC-W: UC model with mesalazine gavage. The relative expression levels of IL-12 (*P* < 0.05), IL-6, IL-17, IL-23, TNF-α, TNFR1 and TNFR2 (*P* < 0.01) in the UC group were increased compared with the HC group. IL-10 (*P* < 0.01) expression levels in the UC group were decreased compared with the HC group. IL-17, IL-23, TNF-α, TNFR2 (*P* < 0.01), IL-6, IL-12, and TNFR1 (*P* < 0.05) expression levels were decreased in the UC-7 group compared with the UC group. TNF-α, TNFR1 (*P* < 0.05), IL-12, IL-6, IL-17, and IL-23 (*P* < 0.01) expression levels in the UC-14 group were decreased compared with UC rats. IL-10 was higher in the UC-7 group (*P* < 0.01), the UC-14 group (*P* < 0.01), and the UC-W group (*P* < 0.05) compared to the UC group. IL-6, IL-17, IL-23, TNF-α, TNFR1, and TNFR2 (*P* < 0.01) expression levels were decreased in the UC-W group compared with the UC group. [a]*P* < 0.05, [b]*P* < 0.01 *vs* the HC group; [c]*P* < 0.05, [d]*P* < 0.01 *vs* the UC group.

increases and IL-10 mRNA ($P < 0.01$) exhibited a significant decrease in the UC rats compared with their expression levels in HC rats (Figure 9). We found significantly lower levels of IL-17, IL-23, TNF-α, TNFR2 ($P < 0.01$), IL-6, IL-12, and TNFR1 mRNA ($P < 0.05$) in the UC-7 group compared to the UC group. The relative expression levels of IL-6, IL-12, IL-17, IL-23 ($P < 0.01$), TNF-α and TNFR1 mRNA ($P < 0.05$) were also lower in the UC-14 group than in the UC group, and IL-10 mRNA was both higher in the UC-7 group ($P < 0.01$), the UC-14 group ($P < 0.01$) and the UC-W group ($P < 0.05$) than in the UC group. However, no significant differences in the relative expression levels of TNFR2 mRNA were noted between the UC-14 and UC groups ($P > 0.05$). Significant decreases in IL-6, IL-17, IL-23, TNF-α, TNFR1, and TNFR2 mRNA ($P < 0.01$) were also noted in the UC-W group compared with the UC group.

The levels of IL-2, IL-10, IL-12, IL-17, IL-23, IFN-γ, LPS, IgA, TGF-β, TNF-α, TNFR1 and TNFR2 in serum

As shown in Figure 10, we noted lower levels of IL-2, IL-10 ($P < 0.01$) and TGF-β ($P < 0.05$) and higher levels of IL-12 ($P < 0.05$), IL-17, IL-23, IFN-γ, LPS, IgA, TNF-α, TNFR1 and TNFR2 ($P < 0.01$) in the UC group compared with HC group. Interestingly, we also noted significantly higher levels of IL-2 ($P < 0.01$), IL-10 (UC-7, $P < 0.05$; UC-W and HC-7, $P < 0.01$) and TGF-β (UC-7 and UC-W, $P < 0.05$; HC-7, $P > 0.05$) in treatment groups than the UC group. Similarly, the levels of IL-17 (UC-7 and HC-7, $P < 0.05$; UC-W, $P < 0.01$), IL-23, IFN-γ, LPS, IgA, TNFR1, TNFR2 ($P < 0.01$), TNF-α ($P < 0.05$) in serum were lower in the UC-7 and UC-W groups. However, there were no significant differences in the level of IL-12 between the UC group and the UC-7 group and between the UC group and the UC-W group ($P > 0.05$). The results were not completely consistent with the PCR. ELISA is a quantitative method to detect proteins, and PCR is the detection of the target gene from the transcript level. Due to post-transcriptional and post-translation regulation, gene expression and protein expression do not necessarily correlate.

DISCUSSION

In the last decade, several studies have revealed a strong association between gut microbiome dysbiosis and UC[17,43-45]. According to the traditional Chinese medicine theory, the Tianshu (ST25) acupoint is the Mu point of large intestine and can regulate gastrointestinal functions, and is usually used to treat abdominal pain and diarrhoea[46]. Moxibustion is an effective and safe treatment for UC, but the underlying mechanism remains unknown. Moxibustion treatment regulates intestinal flora and inhibits TNF-α and IL-12 expression in the colon tissues of UC rats, which can regulate the colonic immune response[30]. However, no study has investigated the effects of moxibustion on the intestinal flora using whole microbiome analysis. Therefore, we employed a 16S rRNA amplicon sequencing approach to determine the effects of moxibustion treatment on the microbiome in a UC rat model.

We found that moxibustion treatment increased Firmicutes abundance and decreased the abundance levels of Bacteroidetes and Proteobacteria. In terms of alpha diversity, HC-14 was more similar to HC than HC-7, which was confirmed by PCoA. In terms of alpha diversity, UC-14 was also more similar to UC than UC-7. This finding suggests that short-term (seven days) but not longer term (fourteen days) moxibustion treatment may significantly affect the gut microbiome. Overall, the relative abundance levels of beneficial bacteria were decreased, and the growth of several opportunistic pathogenic species was increased in the UC rats.

We found that gut microbiome diversity was significantly reduced in UC rats compared with healthy controls, consistent with previous reports. The abundance levels of Firmicutes, Bacteroidetes and Proteobacteria Candidat*us* varied between treatment groups. In particular, Firmicutes was less abundant in UC rats. At the family level, this effect translated to reductions in host beneficial taxa, including *Ruminococcaceae* and *Lactobacillaceae*, which are closely associated with UC[47]. At the genus level, *Lactobacillus*, *Bifidobacterium* and *Ruminococcus* were decreased in UC rats, consistent with the literature[15,46]. Several studies have reported that *Lactobacillus* and *Bifidobacterium* played key roles in treating UC patients or rats by inhibiting Toll-like receptor 4 expression to reduce TNF-α expression[47] and enhancing anti-inflammatory cytokine IL-10 production[48]. UC pathogenesis may be associated with the decreased levels of *Lactobacillus* and *Bifidobacterium*, which consequently led to increases in inflammatory cytokines and decreases in anti-inflammatory cytokines. *Lactobacillus* and *Bifidobacterium* can down-regulate inflammatory cytokines[49] and stimulate anti-inflammatory cytokines, such as IL-10[50], which may protect the host from mucosal inflammation. Interestingly, we found that the expression levels of inflammatory cytokines, such as IL-6, IL-12, IL-17, IL-23, IFN-γ, TNF-α, TNFR1 and TNFR2, were increased and that anti-inflammatory cytokine IL-10 and TGF-β expression was decreased in UC rats compared with HC rats. These results indicate that gut microbial variation may affect mucosal immunity through up-regulating inflammatory cytokines and down-regulating anti-inflammatory cytokines. However, the specific relationship between the gut microbiome and mucosal immunity needs further investigation. After moxibustion treatment, the levels of these inflammatory cytokines were significantly decreased, and anti-inflammatory cytokines were increased. These results demonstrated that moxibustion treatment may repair intestinal mucosal by down-regulating inflammatory cytokines and up-regulating anti-inflammatory cytokines. In an animal model of colitis, butyrate-producing bacteria alleviated intestinal inflammation and necrosis in colitis rats[50]. UC patients have fewer butyrate-producing bacteria, such as *Clostridium leptum* and *Clostridium coccoides*[51,52], which is supported by our findings.
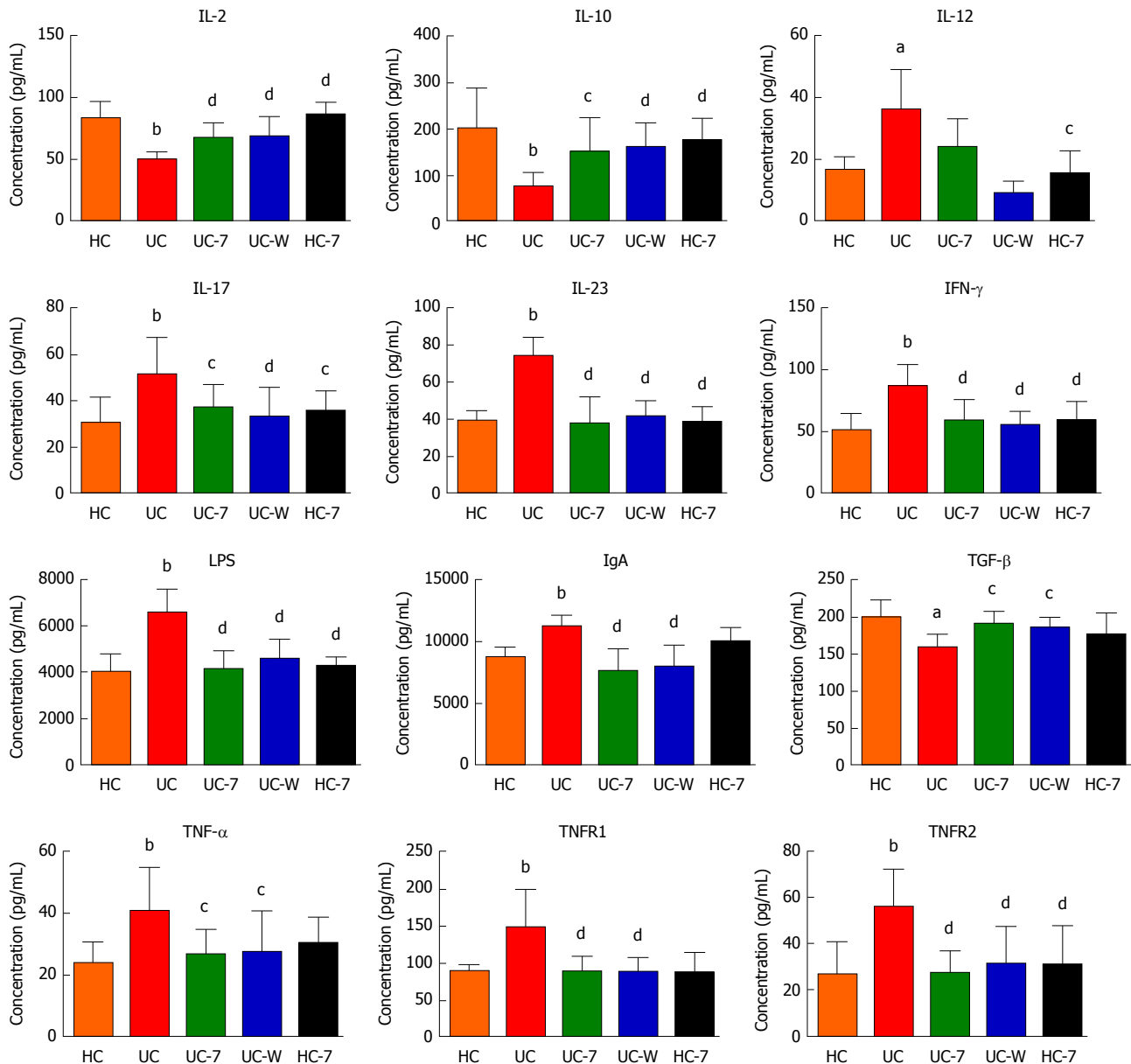
Figure 10 The levels of IL-2, IL-10, IL-12, IL-17, IL-23, IFN-γ, LPS, IgA, TGF-β, TNF-α, TNFR1 and TNFR2 in serum. HC: Healthy controls; UC: UC model group; UC-7: UC model with seven days of moxibustion; UC-W: UC model with mesalazine gavage; HC-7: Healthy controls with seven days of moxibustion. IL-2, IL-10 (P < 0.01) and TGF-β (P < 0.05) were lower and IL-12 (P < 0.05), IL-17, IL-23, IFN-γ, LPS, IgA, TNF-α, TNFR1 and TNFR2 (P < 0.01) were higher in the UC group compared with the HC group. The levels of IL-2 (P < 0.01), IL-10 (UC-7, P < 0.05, UC-W and HC-7, P < 0.01) and TGF-β (UC-7 and UC-W, P < 0.05, HC-7, P > 0.05) were higher in the treatment groups than the UC group. The levels of IL-17 (UC-7 and HC-7, P < 0.05, UC-W, P < 0.01), IL-23, IFN-γ, LPS, IgA, TNFR1, TNFR2 (P < 0.01), and TNF-α (P < 0.05) in serum were lower in the UC-7 and UC-W groups. However, there were no significant differences in the level of IL-12 between the UC group and the UC-7 group and between the UC group and the UC-W group (P > 0.05). [a]P < 0.05, [b]P < 0.01 *vs* the HC group. [c]P < 0.05, [d]P < 0.01 *vs* the UC group.

In UC rats, both moxibustion and mesalazine gavage for seven days led to increases in microbial diversity. Several studies have reported that mesalazine can improve the clinical symptoms and colon mucosal condition in UC patients[53,54]. These results demonstrate that moxibustion could potentially be used as an alternative treatment to mesalazine. It is not clear why the alpha diversity was decreased in UC rats treated with moxibustion for fourteen days. It is possible that moxibustion is not suitable for long-term treatment of UC, and determining whether moxibustion has side effects requires further study. PCoA analysis showed that UC rats had

distinct microbiome profiles compared with HC rats, and an increased similarity in PCoA axis scores was noted between the HC and UC-14 groups. These results indicate that moxibustion could possibly decrease distinct bacteria, such as opportunistic pathogens.

High-throughput sequencing offers a new tool for studying the effect of moxibustion on the gut microbiome and its mechanism of action. Here, we report reduced diversity, gut microbial dysbiosis, increased inflammatory cytokines and decreased anti-inflammatory cytokines in DSS-induced colitis rats and show that these effects could be alleviated by moxibustion treatment. Our findings

suggest that moxibustion exerts its therapeutic effect by modulating the microbiome and intestinal mucosal immunity. Bacterial functions possibly associated with moxibustion treatment include ascorbate and aldarate metabolism and amino acid metabolism.

ARTICLE HIGHLIGHTS

Research background
The aetiology and pathogenesis of ulcerative colitis (UC) are not clear. In recent years, there has been growing evidence that the gut microbiota has a key role in the development of UC. Our previous study has shown that there are an imbalanced states of the beneficial bacteria *Bifidobacterium*, *Lactobacillus*, and harmful bacteria *E. coli* and *Fusobacterium* in the intestine of UC rats, and moxibustion can regulate the balance of beneficial bacteria and harmful bacteria in the intestine of UC rats.

Research motivation
Many studies have confirmed the efficacy of moxibustion in UC, however, the role of gut microbiota in the pathogenesis of UC and the role of moxibustion in the regulation of the UC gut microbiota remain unclear.

Research objectives
The aim of this work is to investigate the effect and mechanism of moxibustion on gut microbiota in UC rats.

Research methods
UC was induced with 4% DSS in drinking water for seven consecutive days. Moxibustion was applied to the Tianshu (ST25, bilateral). Haematoxylin and eosin staining was assessed to evaluate the changes in the colon histopathology. Gut microbiome profiling was conducted by 16S rRNA amplicon sequencing, and PCR and ELISA determined the expression of inflammatory cytokines.

Research results
Compared with the healthy controls (HC) group, the UC group had increased histopathological scores. After treatment, the scores were decreased in the UC-7, UC-14, UC-W, HC-7 and HC-14 groups compared with those in the UC group. The relative abundance of some of the bacteria in the UC group was changed at both the phylum, family and genus level. In addition, UC rats had reduced alpha diversity, which all could be alleviated by moxibustion therapy. Moxibustion can significantly reduce the levels of pro-inflammatory cytokines IL-6, IL-12, IL-17, IL-23, IFN-γ, LPS, TNF-α, TNFR1 and TNFR2 and increase anti-inflammatory cytokines IL-2 and TGF-β in rats with ulcerative colitis and reduce the intestinal inflammation.

Research conclusions
Our findings suggest that moxibustion exerts its therapeutic effect by modulating the microbiome and intestinal mucosal immunity.

Research perspectives
The present study may provide a certain experimental basis and scientific basis for the clinical curative effect of the treatment of UC.

ACKNOWLEDGMENTS

REFERENCES

1   **O'Hara AM**, Shanahan F. The gut flora as a forgotten organ. *EMBO Rep* 2006; **7**: 688-693 [PMID: 16819463 DOI: 10.1038/sj.embor.7400731]

2   **Ubeda C**, Pamer EG. Antibiotics, microbiota, and immune defense. *Trends Immunol* 2012; **33**: 459-466 [PMID: 22677185 DOI: 10.1016/j.it.2012.05.003]

3   **Khan I**, Yasir M, Azhar EI, Kumosani T, Barbour EK, Bibi F, Kamal MA. Implication of gut microbiota in human health. *CNS Neurol Disord Drug Targets* 2014; **13**: 1325-1333 [PMID: 25345506 DOI: 10.2174/1871527313666141023153506]

4   **Clemente JC**, Ursell LK, Parfrey LW, Knight R. The impact of the gut microbiota on human health: an integrative view. *Cell* 2012; **148**: 1258-1270 [PMID: 22424233 DOI: 10.1016/j.cell.2012.01.035]

5   **Sekirov I**, Russell SL, Antunes LC, Finlay BB. Gut microbiota in health and disease. *Physiol Rev* 2010; **90**: 859-904 [PMID: 20664075 DOI: 10.1152/physrev.00045.2009]

6   **Ayres JS**. Cooperative Microbial Tolerance Behaviors in Host-Microbiota Mutualism. *Cell* 2016; **165**: 1323-1331 [PMID: 27259146 DOI: 10.1016/j.cell.2016.05.049]

7   **Zeng MY**, Cisalpino D, Varadarajan S, Hellman J, Warren HS, Cascalho M, Inohara N, Núñez G. Gut Microbiota-Induced Immunoglobulin G Controls Systemic Infection by Symbiotic Bacteria and Pathogens. *Immunity* 2016; **44**: 647-658 [PMID: 26944199 DOI: 10.1016/j.immuni.2016.02.006]

8   **Hooper LV**, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science* 2012; **336**: 1268-1273 [PMID: 22674334 DOI: 10.1126/science.1223490]

9   **Dorrestein PC**, Mazmanian SK, Knight R. Finding the missing links among metabolites, microbes, and the host. *Immunity* 2014; **40**: 824-832 [PMID: 24950202 DOI: 10.1016/j.immuni.2014.05.015]

10   **Baumgart DC**, Sandborn WJ. Inflammatory bowel disease: clinical aspects and established and evolving therapies. *Lancet* 2007; **369**: 1641-1657 [PMID: 17499606 DOI: 10.1016/S0140-6736(07)60751-X]

11   **M'Koma AE**. Inflammatory bowel disease: an expanding global health problem. *Clin Med Insights Gastroenterol* 2013; **6**: 33-47 [PMID: 24833941 DOI: 10.4137/CGast.S12731]

12   **Ng SC**, Lam YT, Tsoi KK, Chan FK, Sung JJ, Wu JC. Systematic review: the efficacy of herbal therapy in inflammatory bowel disease. *Aliment Pharmacol Ther* 2013; **38**: 854-863 [PMID: 23981095 DOI: 10.1111/apt.12464]

13   **Goyal N**, Rana A, Ahlawat A, Bijjem KR, Kumar P. Animal models of inflammatory bowel disease: a review. *Inflammopharmacology* 2014; **22**: 219-233 [PMID: 24906689 DOI: 10.1007/s10787-014-0207-y]

14   **Braun J**, Wei B. Body traffic: ecology, genetics, and immunity in inflammatory bowel disease. *Annu Rev Pathol* 2007; **2**: 401-429 [PMID: 18039105 DOI: 10.1146/annurev.pathol.1.110304.100128]

15   **Michail S**, Durbin M, Turner D, Griffiths AM, Mack DR, Hyams J, Leleiko N, Kenche H, Stolfi A, Wine E. Alterations in the gut microbiome of children with severe ulcerative colitis. *Inflamm Bowel Dis* 2012; **18**: 1799-1808 [PMID: 22170749 DOI: 10.1002/ibd.22860]

16   **Manichanh C**, Borruel N, Casellas F, Guarner F. The gut microbiota in IBD. *Nat Rev Gastroenterol Hepatol* 2012; **9**: 599-608 [PMID: 22907164 DOI: 10.1038/nrgastro.2012.152]

17   **Rajilić-Stojanović M**, Shanahan F, Guarner F, de Vos WM. Phylogenetic analysis of dysbiosis in ulcerative colitis during remission. *Inflamm Bowel Dis* 2013; **19**: 481-488 [PMID: 23385241 DOI: 10.1097/MIB.0b013e31827fec6d]

18   **Kump PK**, Gröchenig HP, Lackner S, Trajanoski S, Reicht G, Hoffmann KM, Deutschmann A, Wenzl HH, Petritsch W, Krejs GJ, Gorkiewicz G, Högenauer C. Alteration of intestinal dysbiosis by fecal microbiota transplantation does not induce remission in patients with chronic active ulcerative colitis. *Inflamm Bowel Dis* 2013; **19**: 2155-2165 [PMID: 23899544 DOI: 10.1097/MIB.0b013e31829ea325]

19   **Machiels K**, Joossens M, Sabino J, De Preter V, Arijs I, Eeckhaut V, Ballet V, Claes K, Van Immerseel F, Verbeke K, Ferrante M, Verhaegen J, Rutgeerts P, Vermeire S. A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. *Gut* 2014; **63**: 1275-1283 [PMID: 24021287 DOI: 10.1136/gutjnl-2013-304833]

20   **Ji J**, Lu Y, Liu H, Feng H, Zhang F, Wu L, Cui Y, Wu H.

Acupuncture and moxibustion for inflammatory bowel diseases: a systematic review and meta-analysis of randomized controlled trials. *Evid Based Complement Alternat Med* 2013; **2013**: 158352 [PMID: 24204388 DOI: 10.1155/2013/158352]

21   **Wu HG**, Zhou LB, Shi DR, Liu SM, Liu HR, Zhang BM, Chen HP, Zhang LS. Morphological study on colonic pathology in ulcerative colitis treated by moxibustion. *World J Gastroenterol* 2000; **6**: 861-865 [PMID: 11819709 DOI: 10.3748/wjg.v6.i6.861]

22   **Joos S**, Wildau N, Kohnen R, Szecsenyi J, Schuppan D, Willich SN, Hahn EG, Brinkhaus B. Acupuncture and moxibustion in the treatment of ulcerative colitis: a randomized controlled study. *Scand J Gastroenterol* 2006; **41**: 1056-1063 [PMID: 16938719 DOI: 10.10 80/00365520600580688]

23   **Mu JP**, Wu HG, Zhang ZQ, Liu HR, Zhu Y, Shi Z, Wang XM. [Meta-analysis on acupuncture and moxibustion for treatment of ulcerative colitis]. *Zhongguo Zhen Jiu* 2007; **27**: 687-690 [PMID: 17926625]

24   **Han Y**, Ma TM, Lu ML, Ren L, Ma XD, Bai ZH. Role of moxibustion in inflammatory responses during treatment of rat ulcerative colitis. *World J Gastroenterol* 2014; **20**: 11297-11304 [PMID: 25170214 DOI: 10.3748/wjg.v20.i32.11297]

25   **Wang X**, Liu Y, Dong H, Wu L, Feng X, Zhou Z, Zhao C, Liu H, Wu H. Herb-Partitioned Moxibustion Regulates the TLR2/NF-κB Signaling Pathway in a Rat Model of Ulcerative Colitis. *Evid Based Complement Alternat Med* 2015; **2015**: 949065 [PMID: 26339273 DOI: 10.1155/2015/949065]

26   **Wu HG**, Huang Z, Liu HR, Zhang W, Shi Y, Zhu Y, Cui YH, Liu SM. [Experimental study on influence of acupuncture and moxibustion therapy on apoptosis of colonic epithelial cells in rats of ulcerative colitis]. *Zhongguo Zhen Jiu* 2005; **25**: 119-122 [PMID: 16312896]

27   **Wu HG**, Liu HR, Tan LY, Gong YJ, Shi Y, Zhao TP, Yi Y, Yang Y. Electroacupuncture and moxibustion promote neutrophil apoptosis and improve ulcerative colitis in rats. *Dig Dis Sci* 2007; **52**: 379-384 [PMID: 17211698 DOI: 10.1007/s10620-006-9561-y]

28   **Huang Y,** Zhao JM, Guan X, Zhang JB, Dou CZ, Shi Z, Liu HR, Wang XM, Wu HG. Effect of Warming Moxibustion on P2RX7 Regulation in Tianshu Acupoint Skin of Rats with Ulcerative Colitis. *Shijie Kexuejishu - Zhongyiyao Xiandaihua* 2016; **18**: 389-394

29   **Zhou EH**, Liu HR, Wu HG, Shi Z, Zhang W, Zhu Y, Shi DR, Zhou S. Down-regulation of protein and mRNA expression of IL-8 and ICAM-1 in colon tissue of ulcerative colitis patients by partition-herb moxibustion. *Dig Dis Sci* 2009; **54**: 2198-2206 [PMID: 19083096 DOI: 10.1007/s10620-008-0620-4]

30   **Wang XM**, Lu Y, Wu LY, Yu SG, Zhao BX, Hu HY, Wu HG, Bao CH, Liu HR, Wang JH, Yao Y, Hua XG, Guo HY, Shen LR. Moxibustion inhibits interleukin-12 and tumor necrosis factor alpha and modulates intestinal flora in rat with ulcerative colitis. *World J Gastroenterol* 2012; **18**: 6819-6828 [PMID: 23239920 DOI: 10.3748/wjg.v18.i46.6819]

31   **Kostic AD**, Xavier RJ, Gevers D. The microbiome in inflammatory bowel disease: current status and the future ahead. *Gastroenterology* 2014; **146**: 1489-1499 [PMID: 24560869 DOI: 10.1053/j.gastro.2014.02.009]

32   **The Ministry of Science and Technology of the People's Republic of China.** Guidance Suggestions for the Care and Use of Laboratory Animals. 2006. Available from: URL: http://www.most.gov.cn/fggw/zfwj/zfwj2006/200609/t20060930_54389.htm

33   **Fichna J**, Dicay M, Lewellyn K, Janecka A, Zjawiony JK, MacNaughton WK, Storr MA. Salvinorin A has antiinflammatory and antinociceptive effects in experimental models of colitis in mice mediated by KOR and CB1 receptors. *Inflamm Bowel Dis* 2012; **18**: 1137-1145 [PMID: 21953882 DOI: 10.1002/ibd.21873]

34   **Weng ZJ**, Wu LY, Zhou CL, Dou CZ, Shi Y, Liu HR, Wu HG. Effect of electroacupuncture on P2X3 receptor regulation in the peripheral and central nervous systems of rats with visceral pain caused by irritable bowel syndrome. *Purinergic Signal* 2015; **11**: 321-329 [PMID: 25809868 DOI: 10.1007/s11302-015-9447-6]

35   **Hamady M**, Walker JJ, Harris JK, Gold NJ, Knight R. Error-correcting barcoded primers for pyrosequencing hundreds of samples

in multiplex. *Nat Methods* 2008; **5**: 235-237 [PMID: 18264105 DOI: 10.1038/nmeth.1184]

36   **Cole JR**, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res* 2009; **37**: D141-D145 [PMID: 19004872 DOI: 10.1093/nar/gkn879]

37   **Langille MG**, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Vega Thurber RL, Knight R, Beiko RG, Huttenhower C. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat Biotechnol* 2013; **31**: 814-821 [PMID: 23975157 DOI: 10.1038/nbt.2676]

38   **Parks DH**, Tyson GW, Hugenholtz P, Beiko RG. STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics* 2014; **30**: 3123-3124 [PMID: 25061070 DOI: 10.1093/bioinformatics/btu494]

39   **Parks DH**, Beiko RG. Identifying biologically relevant differences between metagenomic communities. *Bioinformatics* 2010; **26**: 715-721 [PMID: 20130030 DOI: 10.1093/bioinformatics/btq041]

40   **Lepage P**, Häsler R, Spehlmann ME, Rehman A, Zvirbliene A, Begun A, Ott S, Kupcinskas L, Doré J, Raedler A, Schreiber S. Twin study indicates loss of interaction between microbiota and mucosa of patients with ulcerative colitis. *Gastroenterology* 2011; **141**: 227-236 [PMID: 21621540 DOI: 10.1053/j.gastro.2011.04.011]

41   **Lucke K**, Miehlke S, Jacobs E, Schuppler M. Prevalence of Bacteroides and Prevotella spp. in ulcerative colitis. *J Med Microbiol* 2006; **55**: 617-624 [PMID: 16585651 DOI: 10.1099/jmm.0.46198-0]

42   **Matsuoka K**, Kanai T. The gut microbiota and inflammatory bowel disease. *Semin Immunopathol* 2015; **37**: 47-55 [PMID: 25420450 DOI: 10.1007/s00281-014-0454-4]

43   **Sartor RB**. Microbial influences in inflammatory bowel diseases. *Gastroenterology* 2008; **134**: 577-594 [PMID: 18242222 DOI: 10.1053/j.gastro.2007.11.059]

44   **Trier JS**. Mucosal flora in inflammatory bowel disease: Intraepithelial bacteria or endocrine epithelial cell secretory granules? *Gastroenterology* 2002; **123**: 955; author reply 956 [PMID: 12198731 DOI: 10.1016/S0016-5085(02)70059-0]

45   **Hanning IB,** Lingbeck JM, Ricke SC. Probiotics and Heart Health: Reduction of Risk Factors Associated with Cardiovascular Disease and Complications Due to Foodborne Illnesses. In: Watson RR, Preedy VR, editors. Bioactive Foods in Promoting Health: Probiotics and Prebiotics. Amsterdam: Elsevier Inc, 2010; 423-439 [DOI: 10.1016/B978-0-12-374938-3.00026-8]

46   **Zhang D**, Ren YB, Wu HG, Yang YT, Wu LJ, Zhang J, Shi Z, Ma XP. [Effect of Different Doses of Herbal Cake-partitioned Moxibustion on Histopathological Changes of Colon Tissue in Ulcerative Colitis Rats]. *Zhen Ci Yan Jiu* 2018; **43**: 68-74 [PMID: 29516692 DOI: 10.13702/j.1000-0607.170843]

47   **Yang X**, Fu Y, Liu J, Ren HY. Impact of probiotics on toll-like receptor 4 expression in an experimental model of ulcerative colitis. *J Huazhong Univ Sci Technolog Med Sci* 2013; **33**: 661-665 [PMID: 24142717 DOI: 10.1007/s11596-013-1177-9]

48   **Imaoka A**, Shima T, Kato K, Mizuno S, Uehara T, Matsumoto S, Setoyama H, Hara T, Umesaki Y. Anti-inflammatory activity of probiotic Bifidobacterium: enhancement of IL-10 production in peripheral blood mononuclear cells from ulcerative colitis patients and inhibition of IL-8 secretion in HT-29 cells. *World J Gastroenterol* 2008; **14**: 2511-2516 [PMID: 18442197 DOI: 10.3748/wjg.14.2511]

49   **Llopis M**, Antolin M, Carol M, Borruel N, Casellas F, Martinez C, Espín-Basany E, Guarner F, Malagelada JR. Lactobacillus casei downregulates commensals' inflammatory signals in Crohn's disease mucosa. *Inflamm Bowel Dis* 2009; **15**: 275-283 [PMID: 18839424 DOI: 10.1002/ibd.20736]

50   **Sokol H**, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, Gratadoux JJ, Blugeon S, Bridonneau C, Furet JP, Corthier G, Grangette C, Vasquez N, Pochart P, Trugnan G, Thomas G, Blottière HM, Doré J, Marteau P, Seksik P, Langella P. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad*

Sci USA 2008; **105**: 16731-16736 [PMID: 18936492 DOI: 10.1073/pnas.0804812105]

51   **Andoh A**, Imaeda H, Aomatsu T, Inatomi O, Bamba S, Sasaki M, Saito Y, Tsujikawa T, Fujiyama Y. Comparison of the fecal microbiota profiles between ulcerative colitis and Crohn's disease using terminal restriction fragment length polymorphism analysis. *J Gastroenterol* 2011; **46**: 479-486 [PMID: 21253779 DOI: 10.1007/s00535-010-0368-4]

52   **Manichanh C**, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, Nalin R, Jarrin C, Chardon P, Marteau P, Roca J, Dore J. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut* 2006; **55**: 205-211 [PMID: 16188921 DOI: 10.1136/gut.2005.073817]

53   **Liu B**, Piao X, Guo L, Wang G, Sun W, Gao L, Zheng X, Fang Y. A New Chinese Medicine Intestine Formula Greatly Improves the Effect of Aminosalicylate on Ulcerative Colitis. *Evid Based Complement Alternat Med* 2017; **2017**: 7323129 [PMID: 29358969 DOI: 10.1155/2017/7323129]

54   **Solberg IC**, Lygren I, Jahnsen J, Aadland E, Høie O, Cvancarova M, Bernklev T, Henriksen M, Sauar J, Vatn MH, Moum B; IBSEN Study Group. Clinical course during the first 10 years of ulcerative colitis: results from a population-based inception cohort (IBSEN Study). *Scand J Gastroenterol* 2009; **44**: 431-440 [PMID: 19101844 DOI: 10.1080/00365520802600961]

Basic Study

Integrated genomic analysis for prediction of survival for patients with liver cancer using The Cancer Genome Atlas

Yan-Zhou Song, Xu Li, Wei Li, Zhong Wang, Kai Li, Fang-Liang Xie, Feng Zhang

Yan-Zhou Song, Department of General Surgery, Lianyungang Clinical Medical College of Nanjing Medical University/The First People's Hospital of Lianyungang, Lianyungang 222002, Jiangsu Province, China

Wei Li, Zhong Wang, Kai Li, Fang-Liang Xie, Department of General Surgery, The First People's Hospital of Lianyungang, Lianyungang 222002, Jiangsu Province, China

Xu Li, Feng Zhang, Department of Liver Surgery/Liver Transplantation Center, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

ORCID number: Yan-Zhou Song (0000-0001-9681-7194); Xu Li (0000-0002-3805-0623); Wei Li (0000-0002-6717-676X); Zhong Wang (0000-0001-5821-750X); Kai Li (0000-0003-0095-3066); Fang-Liang Xie (0000-0001-7613-7685); Feng Zhang (0000-0003-3850-467X).

Abstract

AIM
To evaluate the prognostic power of different molecular data in liver cancer.

METHODS
Cox regression screen and least absolute shrinkage and selection operator were performed to select significant prognostic variables. Then the concordance index was calculated to evaluate the prognostic power. For the combination data, based on the clinical cox model, molecular features that better fit the model were combined to calculate the concordance index. Prognostic models were built based on the arithmetic summation of the significant variables. Kaplan-Meier survival curve and log-rank test were performed to compare the survival difference. Then a heatmap was constructed and gene set enrichment analysis was performed for pathway analysis.

RESULTS

The mRNA data were the most informative prognostic variables in all kinds of omics data in liver cancer, with the highest concordance index (C-index) of 0.61. For the copy number variation, methylation and miRNA data, the combination of molecular data with clinical data could significantly boost the prediction accuracy of the molecular data alone ($P < 0.05$). On the other hand, the combination of clinical data with methylation, miRNA and mRNA data could significantly boost the prediction accuracy of the clinical data itself ($P < 0.05$). Based on the significant prognostic variables, different prognostic models were built. In addition, the heatmap analysis, survival analysis, and gene set enrichment analysis validated the practicability of the prognostic models.

CONCLUSION

In all kinds of omics data in liver cancer, the mRNA data might be the most informative prognostic variable. The combination of clinical data with molecular data might be the future direction for cancer prognosis and prediction.

Key words: Liver cancer; Prognosis; Molecular marker; Evaluation; C-index

Core tip: The Cancer Genome Atlas (TCGA) is funded by the National Institute of Health to describe the genomic alterations across cancer types. Several months after the publication of liver cancer TCGA, we systemically evaluated the prognostic power of different omics data of liver cancer. We found that in all kinds of omics data in liver cancer, the mRNA data might be the most informative prognostic variable. The combination of clinical data with molecular data might be the future direction for cancer prognosis and prediction.

INTRODUCTION

Liver cancer is the fourth most common digestive cancer, with the second highest cancer mortality rate worldwide[1,2]. Every year, there are approximately 750,000 new cases, and most of these patients died of liver cancer[2]. Due to tumor heterogeneity and patients' physical status, the prognosis of liver cancer varied across different patients, with the average 5-year survival rate of 26%[2].

Prognostic markers can help make better clinical decision by selecting patients who respond well to some spe-cific treatment. Currently, there are several commonly used clinical markers for liver cancer, such as alpha-fetoprotein (AFP), patient age, tumor stage and some scoring systems[3,4]. In addition, some genetic biomarkers are emerging as novel indicators in cancer diagnosis and prognosis, such as GPC3, DKK1, S100A4, S100A14, SOX6, SUOX, xCT, GRK6, *et al*[5,6].

The Cancer Genome Atlas (TCGA) is funded by the National Institute of Health (NIH) to describe the genomic alterations across different cancer types[7]. It provides tremendous amount of "omics" data, including mRNA sequencing data, miRNA sequencing data, reverse phase protein arrays data, copy number change data and DNA sequencing data. In 2017, the comprehensive genomic characteristics of liver cancer have also been analyzed[8]. It included unsupervised clustering of five molecular platforms to identify the hepatocellular carcinoma patients associated with poor prognosis.

However, there is no consensus on the predictive power of these indicators, especially the molecular markers. Therefore, by utilizing the TCGA data, we aimed to evaluate the prognostic power of liver cancer by molecular markers, and also to assess the predictive power of liver cancer by combining molecular markers and clinical data.

MATERIALS AND METHODS

Data collection and processing

The clinical data and level 3 molecular data, including copy number variation (CNV), methylation, mRNA, miRNA and protein data of the liver cancer patients were downloaded from the TCGA repository (http://gdac.broadinstitute.org/). For the clinical data, we included the patient age and tumor stage, which were the easiest accessible information. For the CNV data, we adopted the Affymetrix genome-wide human SNP array 6.0 platform to detect the copy number variation. After we got the level 3 segmentation file of the copy number data, the GISTIC version 2.0.22 was executed to detect significant regions of amplification and deletion. For the methylation data, the Illumina DNA methylation 450 platform level 3 data was utilized. With respect to the mRNA and miRNA data, the Illumina HiSeq mRNASeq and Illumina HiSeq miRNASeq level 3 data were adopted. For the protein data, we applied the reverse phase protein array (RPPA) data platform.

Concordance index calculation

To evaluate the prognostic power among different omics data, we built a core set of samples. The patients in the core sample set were able to provide complete information from clinical data and different molecular platforms, including CNV, methylation, mRNA, miRNA and protein data. The concordance index (c-index) was calculated according to the method suggested by Yuan *et al*[9]. Briefly, training group patients (80% of total patients) and testing group patients (20% of total

Table 1 Characteristics of the Cancer Genome Atlas samples and molecular platform information

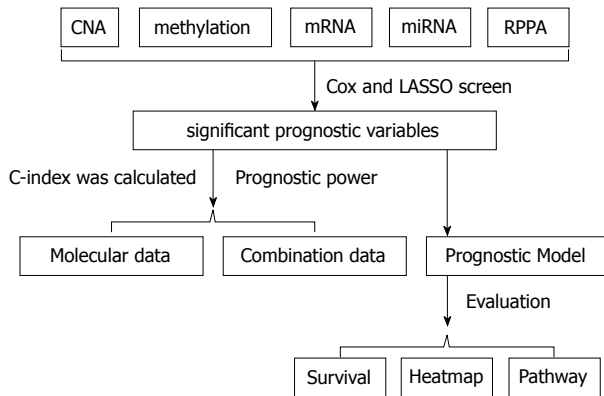|	CNV	Methylation	mRNA	miRNA	Protein
Platform information	SNP_6	450K	HiSeq	HiSeq	RPPA
Sample size	371	377	371	372	184

CNV: Copy number variation.

Figure 1 Statistical process (algorithm). Cox regression screen and least absolute shrinkage and selection operator (LASSO) were performed to select significant prognostic variables. Then the concordance index (C-index) was calculated to evaluate the prognostic power. Prognostic models were built based on the arithmetic summation of the significant variables. Kaplan-Meier survival curve and log-rank test were performed to compare the survival difference. Then the heatmap was constructed and gene set enrichment analysis was performed for pathway analysis.

patients) were randomly divided from the core set for 100 times. Then the cox regression screen was applied to the training group with the R package "survival", in order to select significant prognostic variables. To better converge the training model, least absolute shrinkage and selection operator (LASSO) was performed with the R package "glmnet". Then the produced models were applied to the testing group for prediction. The C-index was calculated 100 times with the R package "survcomp". The Wilcoxon test was utilized to calculate the *P* value ($P < 0.05$ as significant). For the combination data of clinical information and molecular features, clinical variables that were prognostic significant were used to build the cox model. Molecular features that better fit the model were then combined to build a new cox model. Similarly, the c-index was then calculated (Figure 1).

Establishment of prognostic model and survival analysis

To establish the prognostic models, we performed the cox regression analysis and LASSO for all the cancer patients (including both training group and testing group) with complete data. After the significant prognostic variables were identified, we selected the significant variables to build the scoring model. To simplify the process and make it easier to apply in clinic, if the variable value in one patient was higher than the median value, we deemed it to be positive, with the score 1 or -1. Otherwise, the score was 0. The score of 1 or -1 was determined by the corresponding coefficient in the cox

regression analysis. If the coefficient was positive, the score was 1. If the coefficient was negative, the score was -1. Normally, the higher the score was, the higher the risk of poor prognosis. When we divided the patients into the high risk group and low risk group based on the prognostic score, the Kaplan-Meier survival curve was performed to evaluate the overall survival. At the same time, the log-rank test was applied to evaluate the survival difference.

Heatmap construction and gene set enrichment analysis

With respect to the gene expression analysis, the R package "limma" was utilized to detect the differentially expressed genes. Afterwards, the top 100 highly expressed genes and top 100 lowly expressed genes were selected to build the heatmap using the R package "gplots". For the pathway analysis, we performed gene set enrichment analysis (GSEA) proposed by the Broad Institute (http://broadinstitute.org/gsea/downloads. jsp), with the gene sets downloaded from the MSigDB collections (software.broadinstitute.org/gsea/msigdb/ collections.jsp).

RESULTS

Characteristics of the TCGA samples and molecular platform information

Data of the liver cancer patients were downloaded from the TCGA repository (http://gdac.broadinstitute.org/). The platform information and sample size for each data type are shown in Table 1. The mean age of the liver cancer patients was 59 years old. They were 68% male and 32% female. In all of the included patients, 50% were in stage Ⅰ, 25% were in stage Ⅱ, 24% were in stage Ⅲ, and the other 1% were in stage Ⅳ. To evaluate the prognostic power among the different omics data, we also built a core sample set of 171 patients, which only included patients with complete data of all five omics platforms, namely, copy number variation (CNV), methylation, mRNA, miRNA and protein level.

Prognostic power of clinical data and different types of molecular data

The concordance index (C-index) calculated from clinical data and each type of molecular data are shown in Figure 2. The average C-index of clinical data, CNV, methylation, miRNA, mRNA and protein was 0.56, 0.51, 0.57, 0.58, 0.61 and 0.57, respectively. For the molecular data alone, the mRNA data seemed to be the most informative predictors among all molecular data, with the highest C-index of 0.61. The C-index calculated from the
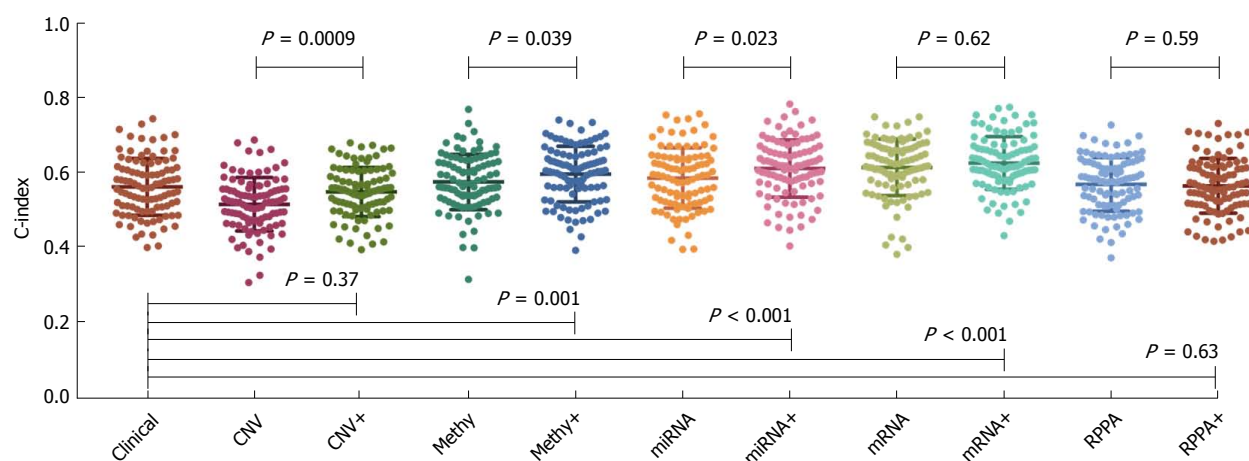
Figure 2 Prognostic powers of clinical data and different types of molecular data. The concordance index (C-index) value on the left Y-axis indicated the prognostic power of each data type. The *P* values on the top half of the figure represented the comparisons between the molecular data alone and the combination data. The *P* values on the lower half of the figure represented the comparisons between the clinical data alone and the combination data.

combination of clinical data and molecular data was 0.55, 0.59, 0.61, 0.62 and 0.56 for the CNV, methylation, miRNA, mRNA and protein data, respectively. In the CNV, methylation and miRNA data, the combination of molecular data with clinical data could significantly boost the prediction accuracy of the molecular data alone (*P* < 0.05). On the other hand, the combination of clinical data with methylation, miRNA and mRNA data could significantly boost the prediction accuracy of the clinical data itself (*P* < 0.05).

Establishment of the prognostic model based on the copy number data

To establish the prognostic model based on the copy number data, we included all 371 patients with complete copy number variation data and clinical data. After the cox regression screen and LASSO, the 10p15.1 and 15q26.3 were identified to be the independent prognostic factors for the liver cancer. Afterwards, the prognostic model based on the significant copy number variation was built, which was able to divide the patients into the high risk group and low risk group (Figure 3A). Kaplan-Meier survival curve showed that there was significant difference with respect to the overall survival between these two group patients (Figure 3B). Afterwards, the limma and GSEA were performed to evaluate the different gene expression and pathways between these two groups. The heatmap showed that there was a distinct gene expression pattern between the high risk group and low risk group (Figure 3C). The GSEA showed that the spermatogenesis, WNT/beta-catenin, E2F targets, mitotic spindle and G2M checkpoint were the top five enriched pathways in the high risk group patients (Figure 3D).

Establishment of the prognostic model based on the methylation data

There were 377 patients with complete methylation data and clinical data that were included in the methylation model. With cox screen and LASSO, REL and MCM2 were shown to be independent prognostic factors. With

a similar method to the copy number variation model, we built the prognostic model based on the methylation data (Figure 4A). Patients with different survival could be distinguished by the prognostic model, as shown in the Kaplan-Meier survival curve (Figure 4B). With respect to the mechanisms leading to the different outcomes, limma and GSEA were performed to show the different gene expression pattern and enriched pathways between the high risk patients and low risk patients (Figure 4C and D). The E2F targets, G2M checkpoint, Myc targets V1, spermatogenesis and PI3K/AKT/mTOR pathway signaling were among the top five enriched pathways in the high risk group of patients (Figure 4D).

Establishment of the prognostic model based on the miRNA data

In the establishment of the miRNA model, there were 372 patients with complete miRNA data and clinical data in the analysis. The cox regression and LASSO were performed to screen the significant prognostic miRNAs. Results showed that miR-3690, miR-561 and miR-621 were independent prognostic factors for the liver cancer patients. Based on these three markers, the prognostic model was built, as shown in Figure 5A. Depending on the scores calculated from the miRNA model, the patients were divided into the high risk group and low risk group. As shown in the Kaplan-Meier survival curve, there was a significant survival difference between these two group patients (Figure 5B). According to the heatmap, it shows distinct gene expression patterns between these group patients (Figure 5C). In addition, GSEA showed that WNT/beta-catenin, G2M checkpoint, allograft rejection, mitotic spindle, and inflammatory response were among the top five enriched pathways in the high risk group patients (Figure 5D).

Establishment of the prognostic model based on the mRNA data

In this section, we included 371 patients with complete mRNA data and clinical data to build the prognostic
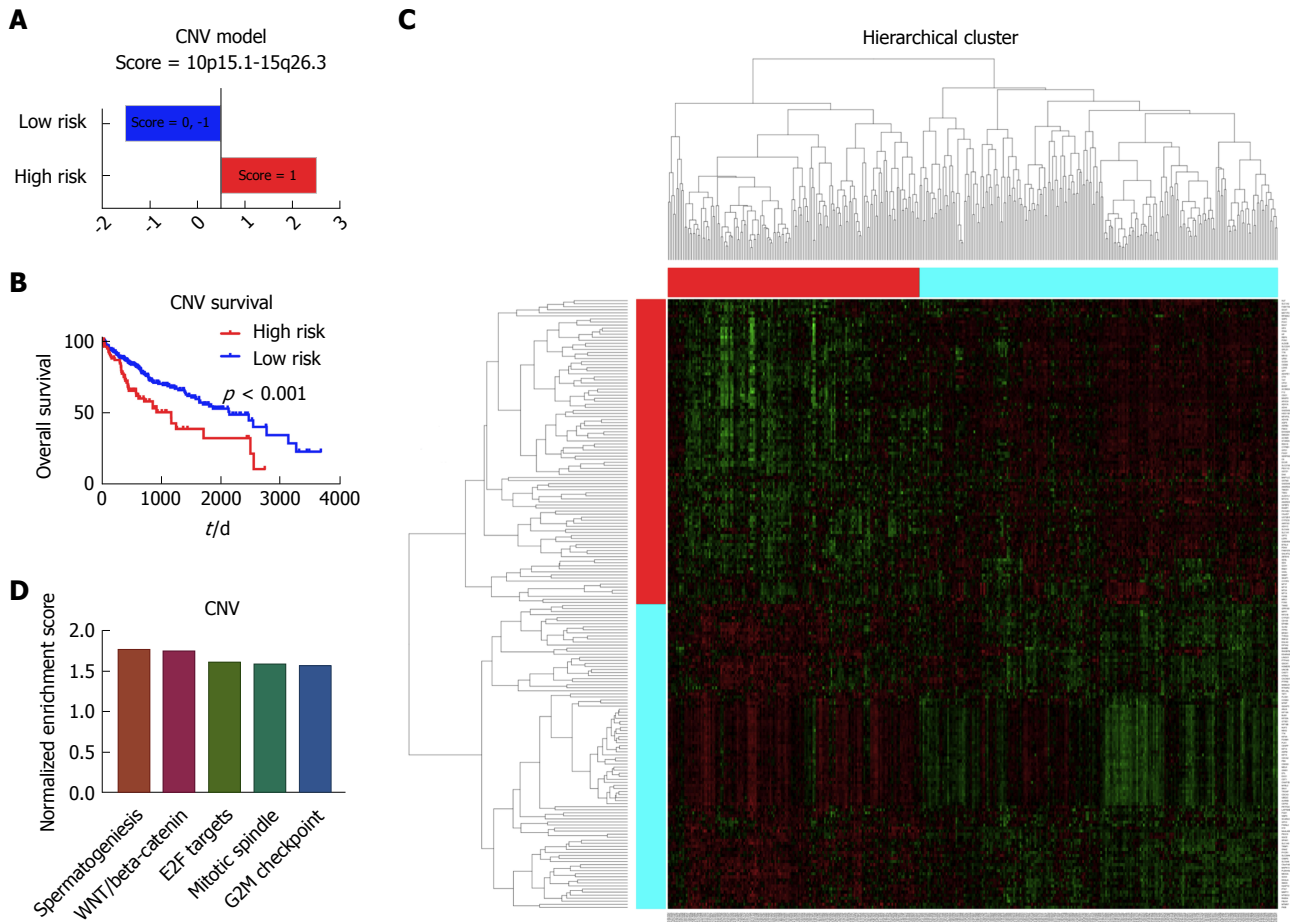
Figure 3 Establishment of prognostic model based on the copy number data. A: The high risk group and low risk group based on the prognostic score. The patients with the score of 1 were considered as high risk, and patients with the score of 0 and -1 were considered as low risk; B: The Kaplan-Meier survival curves of the high risk group and low risk group, which showed that there was significant difference of survival between the high risk patients and low risk patients; C: The heatmap showing the different gene expression patterns of the high risk group and low risk group. It showed that the gene expression patterns of high risk group and low risk group were obviously distinct; D: The top enriched pathways in the high risk group, as indicated by gene set enrichment analysis. It showed that spermatogenesis, WNT/beta-catenin, E2F targets, mitotic spindle and G2M checkpoint were the top five enriched pathways in the high risk group patients.

model. After the cox regression screen and LASSO, CCDC21, GTF3C2, and DBF4 were selected to build the prognostic model, which divided the patients into the high risk group and low risk group based on each patient's prognostic score (Figure 6A). According to the Kaplan-Meier survival curve and log-rank test, there was significant survival difference between these two patient groups (Figure 6B). The heatmap showing different expression patterns of these two group patients is shown in Figure 6C. With respect to the gene set enrichment analysis, the E2F targets, G2M checkpoint, spermatogenesis, mitotic spindle, and Myc targets v1 were significantly enriched in the high risk group patients (Figure 6D).

DISCUSSION

Several months after the publication of liver cancer TCGA, we systemically evaluated the prognostic power of different omics data of liver cancer[8]. We also explored whether additive prognostic power could be gained by the combination of molecular data with clinical data. In

addition, based on the significant prognostic variables identified from the cox regression and LASSO analysis in different omics data, we also built the prognostic models.

In the evaluation of different omics data, we did not include the mutation data from DNA sequencing platform, since the mutation gene and hot-spot mutation site varied a lot among different patients, and it is difficult to divide the patients into a high risk group and low risk group. In addition, it might also need some complicated bioinformatical algorithm to evaluate the prognostic power.

Our results showed that the mRNA data alone seemed to be the most informative prognostic variable (C-index = 0.61) among all the molecular platforms. Consistently, by analyzing breast cancer, glioblastoma multiforme, myeloid leukemia and lung squamous cell carcinoma data, Zhao demonstrated that molecular variables evaluated at the transcription level could reflect patient survival more effectively than those evaluated at the DNA/epigenetic level. There have been several studies focusing on the mRNA prognostic models of liver cancer. For example, Chen *et al*[10] combined three mRNA
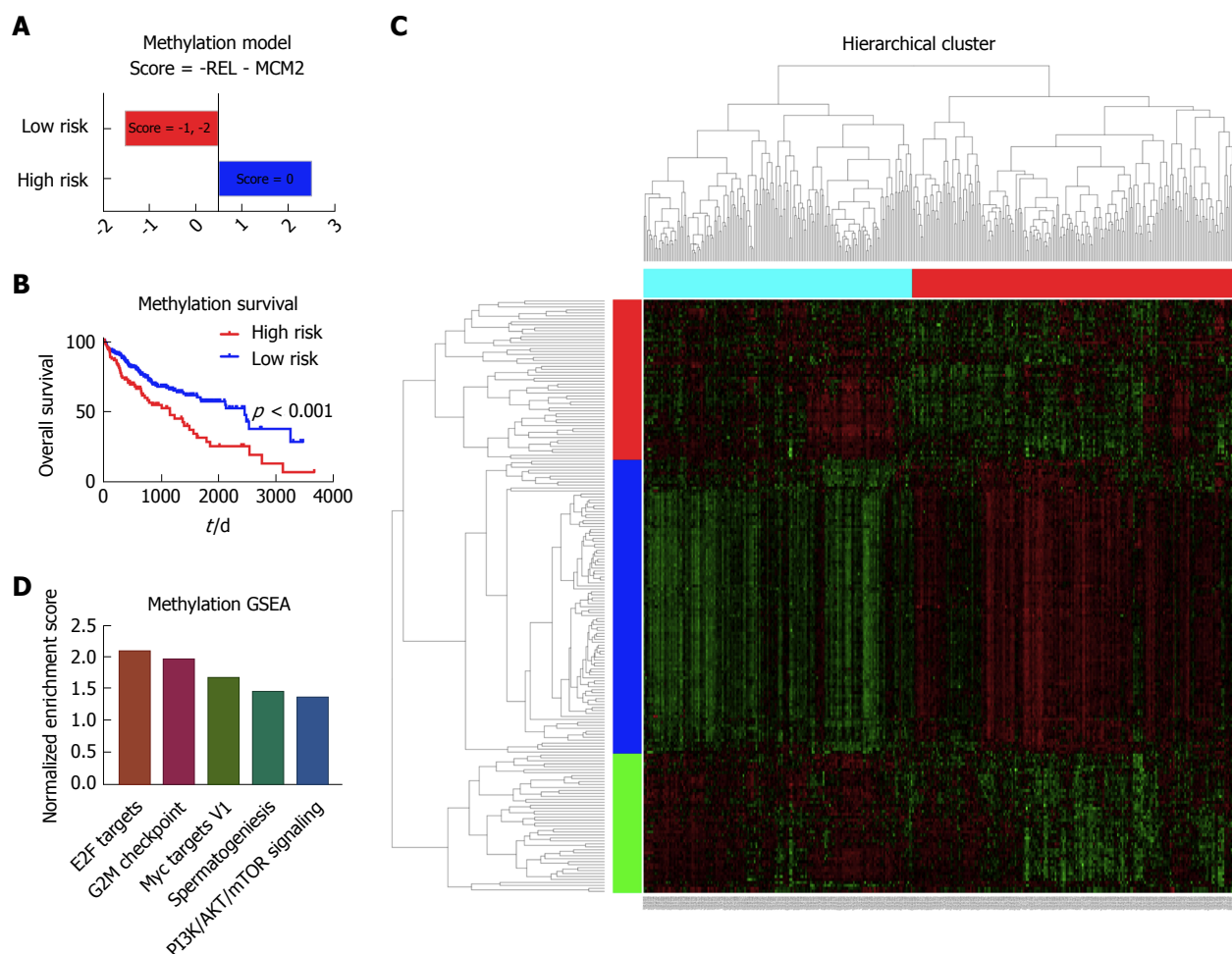
Figure 4 Establishment of the prognostic model based on the methylation data. A: The high risk group and low risk group based on the prognostic score. The patients with the score of 0 were considered as high risk, and patients with the score of -1 and -2 were considered as low risk; B: The Kaplan-Meier survival curves of the high risk group and low risk group, which showed that there was a significant difference of survival between the high risk patients and low risk patients; C: The heatmap showing the different gene expression patterns of the high risk group and low risk group. It showed that the gene expression patterns of high risk group and low risk group were obviously distinct; D: The top enriched pathways in the high risk group, as indicated by the gene set enrichment analysis. It showed that the E2F targets, G2M checkpoint, Myc targets V1, spermatogenesis and PI3K/AKT/mTOR pathway signaling were among the top five enriched pathways in the high risk group of patients.

markers to build the prognostic model and demonstrated it to be useful. Nault *et al*[11] built a 5-gene score associated with overall survival of liver cancer patients after resection. Zhang *et al*[12] defined a hepatic stellate cell 122-gene signature to identify liver cancer patients with poor prognosis. Although with lower prognostic power, some prognostic models based on the CNV, methylation, miRNA and protein data have also widely attracted attention[13-16].

Boulesteix *et al*[17] claimed that little attention was paid to the assessment of the added prognostic power of a molecular signature, given that the clinical predictors or an established model were available. Thus in this study, we showed that the combination of molecular data with clinical data could significantly boost the prediction accuracy of the molecular data alone in the CNV, methylation and miRNA data. On the other hand, the combination of clinical data with methylation, miRNA and mRNA data could significantly boost the prediction accuracy of the clinical data itself. Consistently, Yuan *et al*[9]

summarized that incorporating molecular features with clinical data yielded significantly increased predictions (FDR < 0.05) for kidney cancer, glioblastoma multiforme and ovarian cancer, but the quantitative gains were limited. Some complicated bioinformatical algorithms might be needed to further improve the prognostic power of the combination data.

With respect to the prognostic models based on different omics data, we did not include the protein model, since no significant prognostic variable passed through the cox screen and LASSO analysis. We suppose that one reason is the relative sample size of the patients with protein data. The other reason is due to the complexity of the proteome data. In the copy number model, the 10p15.1 and 15q26.3 were included in our study. Previous studies showed that a telomerase repressor gene might be located on 10p15.1, thus relating it to the prognosis of liver cancer[18]. Meanwhile, genomic copy number variations of 15q26.3 were deemed to be predictive markers for the systemic recurrence of breast
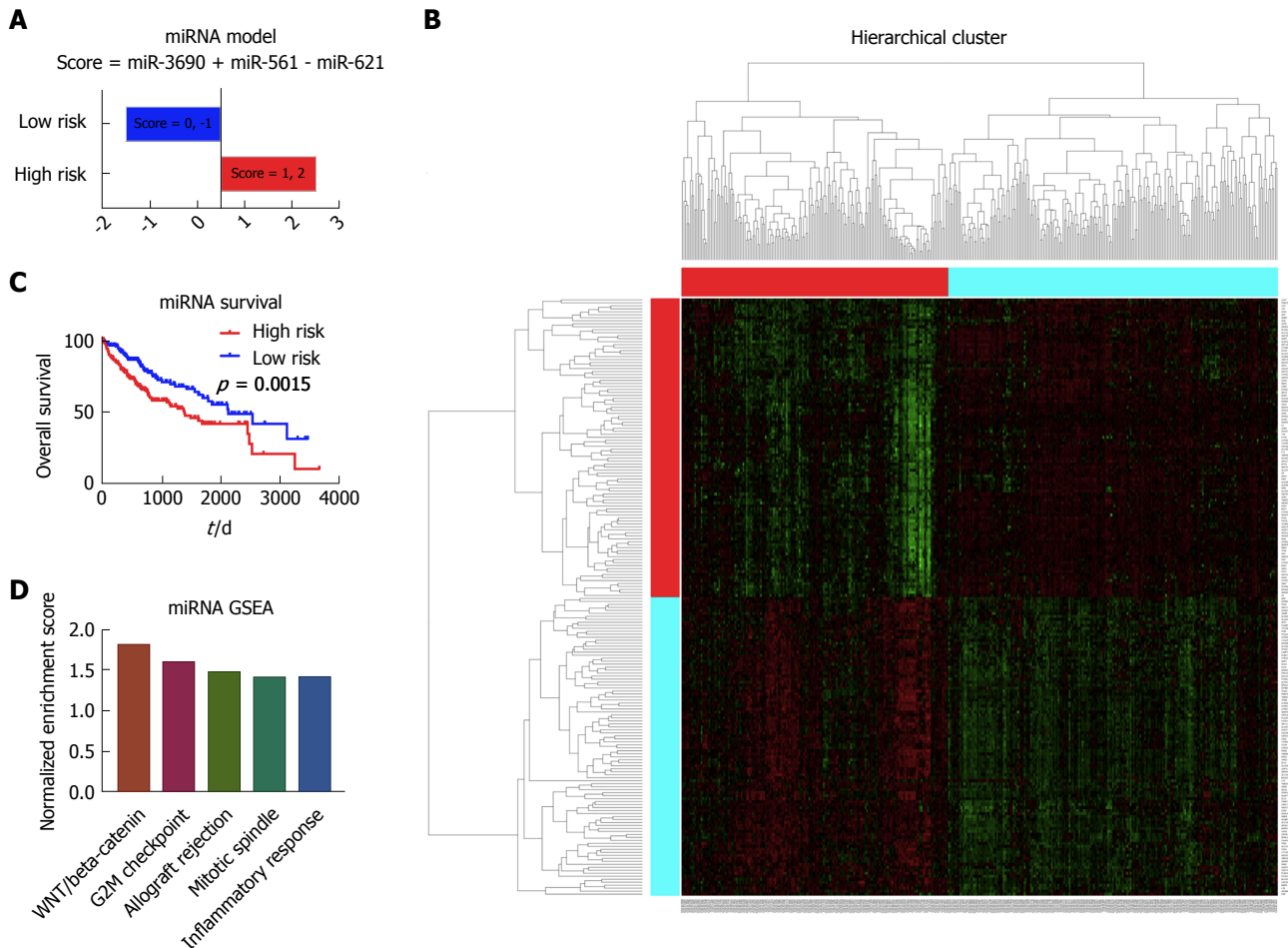
Figure 5 Establishment of the prognostic model based on the miRNA data. A: The high risk group and low risk group based on the prognostic score. The patients with the score of 1 and 2 were considered as high risk, and patients with the score of 0 and -1 were considered as low risk; B: The Kaplan-Meier survival curves of the high risk group and low risk group, which showed that there was significant difference of survival between the high risk patients and low risk patients; C: The heatmap showing the different gene expression patterns of the high risk group and low risk group. It showed that the gene expression patterns of high risk group and low risk group were obviously distinct; D: The top enriched pathways in the high risk group, as indicated by the gene set enrichment analysis. It showed that the WNT/beta-catenin, G2M checkpoint, allograft rejection, mitotic spindle, and inflammatory response were among the top five enriched pathways in the high risk group patients.

cancer[19].

In the analysis of methylation data, REL and MCM2 were demonstrated to be independent prognostic factors. Previous studies showed that REL was involved in apoptosis, inflammation, immune response, and oncogenic processes. Constitutive activation of the Rel/NF-κB pathway could lead to oncogenesis by driving proliferation, enhancing cell survival, or promoting angiogenesis or metastasis[20]. There were also studies reporting that MCM2 was closely related to tumor grade and overall survival in renal cancer[21]. However, this is the first study to demonstrate the methylation of these two markers as prognostic factors in liver cancer.

miRNA alterations were reported to participate in the initiation and progression of human cancer[22]. In our study, miR-3690, miR-561, and miR-621 were included in the prognostic model. This is the first time that we identified miR-3690 as the prognostic variable in cancer prognosis. On the other hand, Qian *et al*[23] reported that miR-561 inhibited cellular proliferation and invasion by targeting c-Myc in gastric cancer. MiR-621 was supposed to sensitize breast cancer to chemotherapy by inhibiting FBXO11 and increasing p53 activity[24]. Thus, it was not difficult to comprehend that they were all involved in the prognosis of liver cancer.

With respect to the mRNA model, CCDC21, GTF3C2 and DBF4 were demonstrated to be independent prognostic factors in liver cancer. CCD21, also called Cep85, is an antagonist of Nek2A that suppresses centrosome disjunction[25]. GTF3C2 is essential for RNA polymerase III-mediated transcription. Until now, there are few studies relating CCD21 and GTF3C2 to cancer progression or prognosis. With respect to DBF4, the CDC7-DBF4 kinase, which was correlated with p53 inactivation, was reported to be overexpressed in multiple cancers[26]. Thus, it is the first study to correlate these markers with patient survival in liver cancer.

Interestingly, most prognostic variables identified in our study seemed to be novel markers in cancer study. Probably due to the strict screen procedure, a lot of
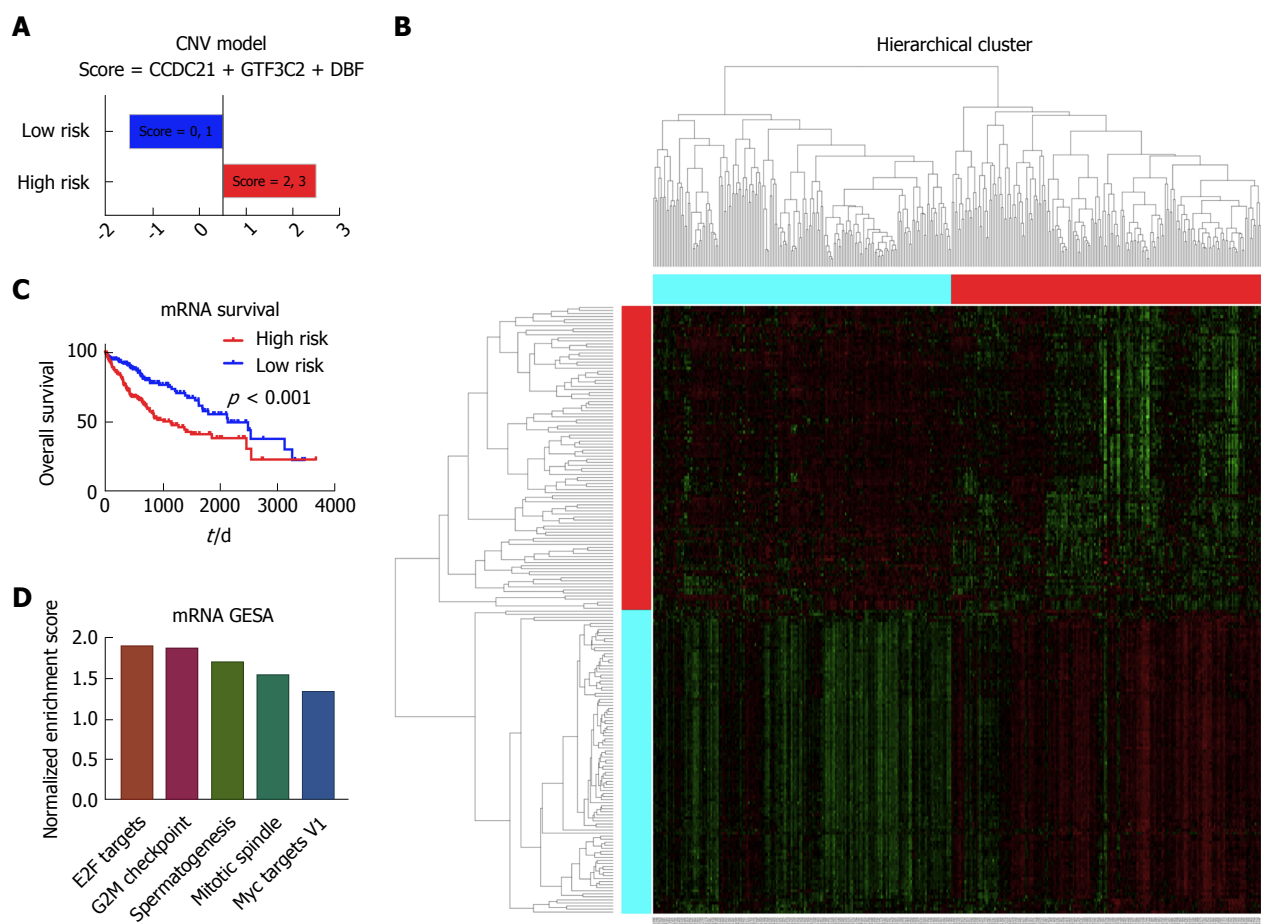
Figure 6 Establishment of the prognostic model based on the mRNA data. A: The high risk group and low risk group based on the prognostic score. The patients with the score of 2 and 3 were considered as high risk, and patients with the score of 0 and 1 were considered as low risk; B: The Kaplan-Meier survival curves of the high risk group and low risk group, which showed that there was significant difference of survival between the high risk patients and low risk patients; C: The heatmap showing the different gene expression patterns of the high risk group and low risk group. It showed that the gene expression patterns of high risk group and low risk group were obviously distinct. D: The top enriched pathways in the high risk group, as indicated by the gene set enrichment analysis. It showed that the E2F targets, G2M checkpoint, spermatogenesis, mitotic spindle, and Myc targets V1 were significantly enriched in the high risk group patients.

traditional prognostic markers were ignored, such as P53 and FOXM1. However, on the other hand, it also demonstrates that our prognostic markers might be more robust and more stable after the strict screening procedure. In summary, the major goal of our study was to evaluate the prognostic power of different omics data in liver cancer. For the first time, we showed that the mRNA data was the most informative prognostic variables in all kinds of omics data in liver cancer. In addition, we also revealed that the combination of clinical data with molecular data might be the future direction for cancer prognosis and prediction. Larger sample size and more mature bioinformatic algorithms were needed to predict the cancer prognosis more precisely in the future.

ARTICLE HIGHLIGHTS

Research background
Liver cancer is the fourth most common digestive cancer worldwide. Prognostic markers can help to make better clinical decision by selecting patients who respond well to some specific treatment. Besides traditional clinical markers, genetic biomarkers are emerging as novel indicators in cancer diagnosis

and prognosis. The Cancer Genome Atlas (TCGA) is funded by the National Institute of Health (NIH) to describe the genomic alterations across cancer types. It provides tremendous amount of "omics" data, including mRNA sequencing, miRNA sequencing, reverse phase protein arrays, copy number change and DNA sequencing.

Research motivation
Although there seems to be great potential value of the clinical and genetic markers, there is no consensus on the predictive power of these indicators, especially the molecular markers.

Research objectives
By utilizing the TCGA data, we aimed to evaluate the prognostic power of liver cancer by molecular markers, and also to assess the predictive power of liver cancer by combining molecular markers and clinical data.

Research methods
Cox regression screen and least absolute shrinkage and selection operator (LASSO) were performed to select significant prognostic variables. Then the concordance index was calculated to evaluate the prognostic power. For the combination data, based on the clinical cox model, molecular features that better fit the model were combined to calculate the concordance index. Prognostic models were built based on the arithmetic summation of the significant variables. Kaplan-Meier survival curve and log-rank test were performed to compare the survival difference. Then the heatmap was constructed and gene set enrichment

analysis was performed for pathway analysis.

Research results

The mRNA data was the most informative prognostic variables in all kinds of omics data in liver cancer. In the copy number variation (CNV), methylation and miRNA data, the combination of molecular data with clinical data could significantly boost the prediction accuracy of the molecular data alone. On the other hand, the combination of clinical data with methylation, miRNA and mRNA data could significantly boost the prediction accuracy of the clinical data itself. Based on the significant prognostic variables, several prognostic models were built. For the CNV data, score = 10p15.1 - 15q26.3. For the methylation data, score = - REL - MCM2. For the miRNA data, score = miR-3690 + miR-561 - miR-621. For the mRNA data, score = CCDC21 + GTF3C2 + DBF4.

Research conclusions

In all kinds of omics data in liver cancer, the mRNA data might be the most informative prognostic variables.

Research perspectives

The combination of clinical data with molecular data might be the future direction for cancer prognosis and prediction.

REFERENCES

1   **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* 2018; **68**: 7-30 [PMID: 29313949 DOI: 10.3322/caac.21442]

2   **Maluccio M**, Covey A. Recent progress in understanding, diagnosing, and treating hepatocellular carcinoma. *CA Cancer J Clin* 2012; **62**: 394-399 [PMID: 23070690 DOI: 10.3322/caac.21161]

3   **Xu XS**, Qu K, Liu C, Zhang YL, Liu J, Song YZ, Zhang P, Liu SN, Chang HL. Highlights for α-fetoprotein in determining prognosis and treatment monitoring for hepatocellular carcinoma. *World J Gastroenterol* 2012; **18**: 7242-7250 [PMID: 23326129 DOI: 10.3748/wjg.v18.i48.7242]

4   **Borzio M**, Dionigi E, Rossini A, Marignani M, Sacco R, De Sio I, Bertolini E, Francica G, Giacomin A, Parisi G, Vicari S, Toldi A, Salmi A, Boccia S, Mitra M, Fornari F. External validation of the ITA.LI.CA prognostic system for patients with hepatocellular carcinoma: A multicenter cohort study. *Hepatology* 2018; **67**: 2215-2225 [PMID: 29165831 DOI: 10.1002/hep.29662]

5   **Zucman-Rossi J**, Villanueva A, Nault JC, Llovet JM. Genetic Landscape and Biomarkers of Hepatocellular Carcinoma. *Gastroenterology* 2015; **149**: 1226-1239.e4 [PMID: 26099527 DOI: 10.1053/j.gastro.2015.05.061]

6   **Scaggiante B**, Kazemi M, Pozzato G, Dapas B, Farra R, Grassi M, Zanconati F, Grassi G. Novel hepatocellular carcinoma molecules with prognostic and therapeutic potentials. *World J Gastroenterol* 2014; **20**: 1268-1288 [PMID: 24574801 DOI: 10.3748/wjg.v20.i5.1268]

7   **Tomczak K**, Czerwińska P, Wiznerowicz M. The Cancer Genome Atlas (TCGA): an immeasurable source of knowledge. *Contemp Oncol* (Pozn) 2015; **19**: A68-A77 [PMID: 25691825 DOI: 10.5114/wo.2014.47136]

8   **Cancer Genome Atlas Research Network**; Cancer Genome Atlas Research Network. Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma. *Cell* 2017; **169**: 1327-1341.e23 [PMID: 28622513 DOI: 10.1016/j.cell.2017.05.046]

9   **Yuan Y**, Van Allen EM, Omberg L, Wagle N, Amin-Mansour A, Sokolov A, Byers LA, Xu Y, Hess KR, Diao L, Han L, Huang X, Lawrence MS, Weinstein JN, Stuart JM, Mills GB, Garraway LA, Margolin AA, Getz G, Liang H. Assessing the clinical utility of cancer genomic and proteomic data across tumor types. *Nat Biotechnol* 2014; **32**: 644-652 [PMID: 24952901 DOI: 10.1038/nbt.2940]

10 **Chen SS**, Yu KK, Ling QX, Huang C, Li N, Zheng JM, Bao SX, Cheng Q, Zhu MQ, Chen MQ. The combination of three molecular markers can be a valuable predictive tool for the prognosis of

11 **Nault JC**, De Reyniès A, Villanueva A, Calderaro J, Rebouissou S, Couchy G, Decaens T, Franco D, Imbeaud S, Rousseau F, Azoulay D, Saric J, Blanc JF, Balabaud C, Bioulac-Sage P, Laurent A, Laurent-Puig P, Llovet JM, Zucman-Rossi J. A hepatocellular carcinoma 5-gene score associated with survival of patients after liver resection. *Gastroenterology* 2013; **145**: 176-187 [PMID: 23567350 DOI: 10.1053/j.gastro.2013.03.051]

12 **Zhang DY**, Goossens N, Guo J, Tsai MC, Chou HI, Altunkaynak C, Sangiovanni A, Iavarone M, Colombo M, Kobayashi M, Kumada H, Villanueva A, Llovet JM, Hoshida Y, Friedman SL. A hepatic stellate cell gene expression signature associated with outcomes in hepatitis C cirrhosis and hepatocellular carcinoma after curative resection. *Gut* 2016; **65**: 1754-1764 [PMID: 26045137 DOI: 10.1136/gutjnl-2015-309655]

13 **Kawaguchi K**, Honda M, Yamashita T, Okada H, Shirasaki T, Nishikawa M, Nio K, Arai K, Sakai Y, Yamashita T, Mizukoshi E, Kaneko S. Jagged1 DNA Copy Number Variation Is Associated with Poor Outcome in Liver Cancer. *Am J Pathol* 2016; **186**: 2055-2067 [PMID: 27315779 DOI: 10.1016/j.ajpath.2016.04.011]

14 **Villanueva A**, Portela A, Sayols S, Battiston C, Hoshida Y, Méndez-González J, Imbeaud S, Letouzé E, Hernandez-Gea V, Cornella H, Pinyol R, Solé M, Fuster J, Zucman-Rossi J, Mazzaferro V, Esteller M, Llovet JM; HEPTROMIC Consortium. DNA methylation-based prognosis and epidrivers in hepatocellular carcinoma. *Hepatology* 2015; **61**: 1945-1956 [PMID: 25645722 DOI: 10.1002/hep.27732]

15 **Barry CT**, D'Souza M, McCall M, Safadjou S, Ryan C, Kashyap R, Marroquin C, Orloff M, Almudevar A, Godfrey TE. Micro RNA expression profiles as adjunctive data to assess the risk of hepatocellular carcinoma recurrence after liver transplantation. *Am J Transplant* 2012; **12**: 428-437 [PMID: 22008552 DOI: 10.1111/j.1600-6143.2011.03788.x]

16 **Tan GS**, Lim KH, Tan HT, Khoo ML, Tan SH, Toh HC, Ching Ming Chung M. Novel proteomic biomarker panel for prediction of aggressive metastatic hepatocellular carcinoma relapse in surgically resectable patients. *J Proteome Res* 2014; **13**: 4833-4846 [PMID: 24946162 DOI: 10.1021/pr500229n]

17 **Boulesteix AL**, Sauerbrei W. Added predictive value of high-throughput molecular data to clinical data and its validation. *Brief Bioinform* 2011; **12**: 215-229 [PMID: 21245078 DOI: 10.1093/bib/bbq085]

18 **Leuraud P**, Aguirre-Cruz L, Hoang-Xuan K, Crinière E, Tanguy ML, Golmard JL, Kujas M, Delattre JY, Sanson M. Telomerase reactivation in malignant gliomas and loss of heterozygosity on 10p15.1. *Neurology* 2003; **60**: 1820-1822 [PMID: 12796538 DOI: 10.1212/01.WNL.0000065894.89082.5E]

19 **Hwang KT**, Han W, Cho J, Lee JW, Ko E, Kim EK, Jung SY, Jeong EM, Bae JY, Kang JJ, Yang SJ, Kim SW, Noh DY. Genomic copy number alterations as predictive markers of systemic recurrence in breast cancer. *Int J Cancer* 2008; **123**: 1807-1815 [PMID: 18649361 DOI: 10.1002/ijc.23672]

20 **Gilmore T**, Gapuzan ME, Kalaitzidis D, Starczynowski D. Rel/NF-kappa B/I kappa B signal transduction in the generation and treatment of human cancer. *Cancer Lett* 2002; **181**: 1-9 [PMID: 12430173 DOI: 10.1016/S0304-3835(01)00795-9]

21 **Dudderidge TJ**, Stoeber K, Loddo M, Atkinson G, Fanshawe T, Griffiths DF, Williams GH. Mcm2, Geminin, and KI67 define proliferative state and are prognostic markers in renal cell carcinoma. *Clin Cancer Res* 2005; **11**: 2510-2517 [PMID: 15814627 DOI: 10.1158/1078-0432.CCR-04-1776]

22 **Calin GA**, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006; **6**: 857-866 [PMID: 17060945 DOI: 10.1038/nrc1997]

23 **Qian K**, Mao B, Zhang W, Chen H. MicroRNA-561 inhibits gastric cancercell proliferation and invasion by downregulating c-Myc expression. *Am J Transl Res* 2016; **8**: 3802-3811 [PMID: 27725860]

24 **Xue J**, Chi Y, Chen Y, Huang S, Ye X, Niu J, Wang W, Pfeffer LM,

Shao ZM, Wu ZH, Wu J. MiRNA-621 sensitizes breast cancer to chemotherapy by suppressing FBXO11 and enhancing p53 activity. *Oncogene* 2016; **35**: 448-458 [PMID: 25867061 DOI: 10.1038/onc.2015.96]

25   **Chen C**, Tian F, Lu L, Wang Y, Xiao Z, Yu C, Yu X. Characterization of Cep85 - a new antagonist of Nek2A that is involved in the regulation of centrosome disjunction. *J Cell Sci* 2015; **128**: 3290-3303 [PMID: 26220856 DOI: 10.1242/jcs.171637]

26   **Bonte D**, Lindvall C, Liu H, Dykema K, Furge K, Weinreich M. Cdc7-Dbf4 kinase overexpression in multiple cancers and tumor cell lines is correlated with p53 inactivation. *Neoplasia* 2008; **10**: 920-931 [PMID: 18714392 DOI: 10.1593/neo.08216]

Retrospective Study

Multikinase inhibitor-associated hand-foot skin reaction as a predictor of outcomes in patients with hepatocellular carcinoma treated with sorafenib

Masanori Ochi, Toshiro Kamoshida, Atsushi Ohkawara, Haruka Ohkawara, Nobushige Kakinoki, Shinji Hirai, Akinori Yanaka

Masanori Ochi, Toshiro Kamoshida, Atsushi Ohkawara, Haruka Ohkawara, Nobushige Kakinoki, Shinji Hirai, Department of Gastroenterology, Hitachi General Hospital, Ibaraki 317-0077, Japan

Akinori Yanaka, Hitachi Medical Education and Research Center, University of Tsukuba, Ibaraki 317-0077, Japan

ORCID number: Masanori Ochi (0000-0003-0850-6076); Toshiro Kamoshida (0000-0003-4930-1018); Atsushi Ohkawara (0000-0003-1764-565X); Haruka Ohkawara (0000-0001-8427-5826); Nobushige Kakinoki (0000-0002-1522-2226); Shinji Hirai (0000-0001-7141-0240); Akinori Yanaka (0000-0001-7967-5665).

Abstract

AIM

To investigate the relationship between the onsets of multikinase inhibitor (MKI)-associated hand-foot skin reaction (HFSR) and prognosis under intervention by pharmacists after the introduction of sorafenib.

METHODS

We conducted a retrospective study involving 40 patients treated with sorafenib. Intervention by pharmacists began at the time of treatment introduction and continued until the appearance of symptomatic exacerbation or non-permissible adverse reactions. We examined the relationship between MKI-associated HFSR and overall survival (OS) after the initiation of treatment.

RESULTS

The median OS was 10.9 mo in the MKI-associated HFSR group and 3.4 mo in the no HFSR group, showing a significant difference in multivariate analysis. A multivariate analysis of the time to treatment failure indicated that the intervention by pharmacists and MKI-associated HFSR were significant factors. The median cumulative dose and the mean medication possession ratio were significantly higher in the intervention group than in the non-intervention group. A borderline significant difference was observed in terms of OS in this group.

CONCLUSION

Intervention by pharmacists increased drug adherence. Under increased adherence, MKI-associated HFSR was an advantageous surrogate marker. Intervention by healthcare providers needs to be performed for adequate sorafenib treatment.

Key words: Hepatocellular carcinoma; Surrogate marker; Multikinase inhibitor-associated hand-foot skin reaction; Sorafenib; Intervention by pharmacists

Core tip: Sorafenib is an oral anticancer drug associated with a high incidence of adverse reactions. However, no studies have evaluated its therapeutic efficacy under improved adherence. A surrogate marker of significant improvement in overall survival under improved adherence in advanced hepatocellular carcinoma patients after the introduction of sorafenib was multikinase inhibitor-associated hand-foot skin reaction. Intervention by healthcare providers, including pharmacists specializing in cancer treatment, has improved patient adherence, contributing to the true response to sorafenib treatment.

INTRODUCTION

A double-blind, randomized phase Ⅲ study of sorafenib therapy for advanced hepatocellular carcinoma was previously conducted. The time to progression (TTP) and overall survival (OS) were significantly longer in the sorafenib-treated group than in the placebo group[1-4]. In addition, randomized phase Ⅲ studies using sunitinib or brivanib have been performed, and no significant differences were observed from sorafenib[5,6]. A randomized phase Ⅲ study using regorafenib demonstrated that this drug significantly prolonged OS compared with that in the placebo group; however, there is currently no evidence to support regorafenib as a first-choice drug[7].

A surrogate marker that reflects a better prognosis after the introduction of sorafenib has not yet been established[8]. Previous studies have reported a relationship between multikinase inhibitor (MKI)-associated hand-foot skin reaction (HFSR) and TTP/OS[9-12]. Sorafenib frequently induces adverse events (AEs). The most frequent AE is MKI-associated HFSR[1-4]. Although MKI-associated HFSR is not life-threatening, its exacerbation reduces the quality of life of patients, which affects adherence to sorafenib treatments. A previous study indicated that intervention by nurses improved the efficacy of sorafenib treatments[13]; however, intervention by pharmacists who are familiar with sorafenib treatments has not yet been investigated.

The purpose of the present study was to clarify the relationship between therapeutic efficacy and MKI-associated HFSR in sorafenib-treated patients with advanced hepatocellular carcinoma. We also examined the influence of intervention by pharmacists on adherence.

MATERIALS AND METHODS

Sorafenib therapy

This retrospective study was conducted on patients treated between May 2009 and March 2017 at our institution based on the Barcelona Clinic Liver Cancer (BCLC) strategy[14,15]. Indication criteria for sorafenib included the following: (1) Child-Pugh grade, A or B; (2) Eastern Cooperative Oncology Group Performance Status, 0 or 1; (3) hemoglobin level, $\geqslant$ 8.5 g/dL; (4) neutrophil count, > 1500/μL; (5) platelet count, > 75000/μL; (6) total bilirubin level, < 2.0 mg/dL; (7) alanine and aminotransferases, < 5-fold the upper limit of the normal range; and (8) no necessity for dialysis. Exclusion criteria included the following: (1) A history of serious hypersensitivity to the components of this drug; (2) pregnant women or those who may be pregnant; (3) a history of thrombosis or ischemic heart disease; and (4) brain metastasis. The initial dose of sorafenib was established as 200, 400, or 800 mg, but it was adjusted according to the criteria for dose reductions described in the package inserts or matters recommended by pharmacists. Administration was continued until the appearance of symptomatic exacerbation or non-permissible AEs.

Written informed consent was obtained from all subjects. The protocol of this study was approved by the Institutional Review Board of our hospital and was performed according to the Helsinki Declaration.

Clinical evaluation

The treatment response was evaluated using computed tomography or magnetic resonance imaging at 8-wk intervals. Radiological evaluations were conducted based on the mRECIST criteria[16]. The National Cancer Institute Common Toxicity Criteria for Adverse Events (NCI-CTCAE)

Table 1 Baseline characteristics of patients

Patient features	n (%)
Total No. of patients	40
Age (yr)	
Median (range)	71 (48-89)
Sex	
Male	31 (78)
Female	9 (12)
Child-Pugh class	
A	36 (90)
B	4 (10)
Underlying cause	
HCV	23 (58)
HBV	10 (25)
Others	7 (17)
Portal vein thrombosis	11 (28)
Liver only	26 (60)
Metastatic disease	24 (40)
AFP (ng/mL)	
> 100	26 (65)
≤ 100	14 (35)
DCP (mAU/mL)	
> 1000	19 (48)
≤ 1000	21 (52)
BCLC stage	
B	16 (40)
C	24 (60)
Intervention by pharmacists	
Yes	22 (55)
No	18 (45)

AFP: Alpha-fetoprotein; BCLC: Barcelona Clinic Liver Cancer; DCP: Des-gamma-carboxy prothrombin; HCV: Hepatitis C virus; HBV: Hepatitis B virus.

(version 4.0) were adopted to assess AEs. Patients were divided into two groups: Patients who had developed grade 1 or higher MKI-associated HFSR and those who had not.

Intervention by pharmacists and the double-check system

The Pharmacists' Outpatient Clinic (primary outpatient clinic) was established through intervention by pharmacists specializing in cancer treatment. At the primary outpatient clinic, AE assessment/management education for patients and residual drug/self-management education were conducted at 2-wk intervals (average). The duration of a consultation at this outpatient clinic per session was 20 min to 30 min. We adopted a system to summarize the results of the consultation at the primary outpatient clinic as a report and advised physicians responsible for outpatient care (secondary outpatient clinic). In addition, when grade 3 or higher AEs occurred, a prompt management system to telephone or directly speak with physicians at the secondary outpatient clinic in addition to reporting was established. At the secondary outpatient clinic, physicians responsible for outpatient care examined patients based on patient information obtained at the primary outpatient clinic and made a final decision on the administration of sorafenib. A system for physicians at the secondary outpatient clinic to promptly consult specialists belonging to each department at the onset of AEs was established. Regarding care for patient

anxiety, nurses specializing in cancer nursing arranged an interview- or telephone-based back-up support system. Intervention by pharmacists specializing in cancer treatments began at the time of treatment introduction and continued until the appearance of symptomatic exacerbation or non-permissible AEs.

Statistical analysis

In the survival analysis, the significance of differences in survival between two groups with and without MKI-associated HFSR after the administration of sorafenib was tested using the log-rank test[17]. The log-rank test was also used to compare survival between two groups with and without intervention by pharmacists. In the multivariate analysis, Cox proportional hazards model was adopted[18]. Regarding the presence or absence of MKI-associated HFSR and antitumor effects, a landmark analysis[19] was conducted to minimize time-dependent bias. AE-free patients 28 d after the start of treatment, when the highest-grade MKI-associated HFSR was noted in ≥ 50% of the patients, was analyzed as a landmark.

Fisher's exact test was used for categorical variables. The Mann-Whitney *U*-test was adopted for continuous variables. SPSS software (version 22, SPSS, Inc., Chicago, IL, United States) was used for statistical analysis. A *P*-value of 0.05 considered as significant.

RESULTS

Baseline characteristics

Sorafenib treatment was evaluated in 40 patients treated between May 2009 and March 2017. The baseline characteristics of these patients are shown in Table 1. The median age was 71 years (range: 48-89 years). Thirty-nine patients (97.5%) underwent sorafenib treatment after hepatectomy, radiofrequency ablation, or trans-catheter arterial chemoembolization (TACE), which was ineffective. The most frequent etiological factor for hepatitis was hepatitis C virus (57.5%), followed by hepatitis B virus (25%). Extrahepatic metastases were detected in 16 patients (40%), while tumors were localized in the liver in the other 24 patients (60%). Twenty-two patients (55%) had received intervention by pharmacists.

AEs

AEs related to sorafenib are presented in Table 2. In all patients, ≥ 1 AE was observed, but there were no grade 4 AEs. Primary AEs consisted of MKI-associated HFSR (55%), anemia (35%), hypertension (30%), anorexia (30%), and thrombopenia (30%). The grades of these AEs were evaluated as 1 in 38%, 2 in 35%, and 3 in 28% of patients. In 16 patients, treatments were postponed (definitive discontinuation) due to AEs. In 11 patients, the dose was reduced after definitive discontinuation.

Treatment efficacy

The relationship between OS and MKI-associated HFSR is shown in Figure 1. The median OS was 222 d (7.4 mo) (95%CI: 175-268), range: 14-618 d). In the presence

Table 2 Adverse events according to Common Toxicity Criteria for Adverse Events v4.0 *n* (%)

Adverse events	Grade 1	Grade 2	Grade 3	All
MKI-associated HFSR	11 (27)	6 (15)	5 (13)	22 (55)
Anemia	10 (25)	4 (10)	0 (0)	14 (35)
Hypertension	7 (17)	5 (13)	0 (0)	12 (30)
Anorexia	7 (17)	4 (10)	1 (3)	12 (30)
Decreased platelet count	4 (10)	4 (10)	4 (10)	12 (30)
Alopecia	9 (22)	1 (3)	0 (0)	10 (25)
Diarrhea	5 (13)	0 (0)	2 (4)	7 (17)

MKI-associated HFSR: Multikinase inhibitor-associated hand-foot skin reaction.

Table 3 Univariate and multivariate analyses of predictive factors for overall survival

	Univariate analysis		Multivariate analysis		
	HR	*P* value	HR	*P* value	95%CI
Age (≥ 65 yr)	0.494	0.079			
Initial dose (800 mg)	1.635	0.182			
Early AFP response	0.834	0.622			
AFP (< 100 ng/dL)	0.756	0.450			
DCP (< 1000 mAU/mL)	0.806	0.554			
MKI-associated HFSR	0.224	< 0.001	0.241	0.001	0.102-0.567
BCLC B	0.358	0.013	0.404	0.041	0.170-0.964
Hypertension	0.889	0.756			
Diarrhea	0.950	0.911			
Anorexia	1.671	0.162			
Alopecia	0.560	0.177			

Early alpha-fetoprotein (AFP) response refers to a ≥ 20% improvement in AFP levels within 2 mo. BCLC: Barcelona Clinic Liver Cancer; DCP: Des-gamma-carboxy prothrombin; MKI-associated HFSR: Multikinase inhibitor-associated hand-foot skin reaction.

Table 4 Univariate and multivariate analyses of variables associated with time to treatment failure

	Univariate analysis		Multivariate analysis		
	HR	*P* value	HR	*P* value	95%CI
Age (≥ 65 yr)	0.369	0.020	0.475	0.099	0.196-1.150
Initial dose (800 mg)	1.213	0.588			
Early AFP response	0.630	0.24			
AFP (< 100 ng/dL)	0.988	0.974			
DCP (< 1000 mAU/mL)	0.711	0.363			
MKI-associated HFSR	0.334	0.004	0.418	0.048	0.175-0.994
BCLC B	0.970	0.932			
Hypertension	1.286	0.510			
Diarrhea	0.517	0.159			
Anorexia	1.652	0.175			
Alopecia	0.337	0.019	0.792	0.679	0.262-2.391
Intervention by pharmacists	0.326	0.002	0.425	0.042	0.186-0.971

Early alpha-fetoprotein (AFP) response refers to a ≥ 20% improvement in AFP levels within 2 mo. BCLC: Barcelona Clinic Liver Cancer; DCP: Des-gamma-carboxy prothrombin; MKI-associated HFSR: Multikinase inhibitor-associated hand-foot skin reaction.

of grade 1 or higher MKI-associated HFSR, OS was significantly prolonged. The results of the univariate- and multivariate analyses regarding OS are presented in Table 3. A multivariate analysis was conducted on factors with *P*-values of < 0.05 in the univariate analysis. The results of the multivariate analysis showed that the presence of MKI-associated HFSR [hazard ratio (HR) = 0.241, 95%CI: 0.102-0.567; *P* = 0.001] and BCLC B (HR = 0.404, 95%CI: 0.170-0.964; *P* = 0.041) were significant predictive factors for the prolongation of OS. No significant differences were observed in the rate of

decreased alpha-fetoprotein (AFP) levels in the early stage, diarrhea, or hypertension. A borderline significant difference was observed in terms of OS between patients with and without intervention by pharmacists (5.2 mo in the non-intervention group *vs* 9.7 mo in the intervention group, *P* = 0.097). The disease control rate was 33% [partial response: 1 patient (3%); stable disease: 12 patients (30%)].

Treatment and intervention by pharmacists
The median time to treatment failure (TTF) was 65 d

Figure 1 Kaplan-Meier survival plot for overall survival in multikinase inhibitor-associated hand-foot skin reaction (landmark analysis).

No. at risk							
≥ Grade 1	22	20	15	9	5	2	1
No skin reaction	17	10	5	1	0	0	0

(2.2 mo) (95% CI: 44-86, range: 7-347 d). The median cumulative dose was 20000 mg (range: 2800-146400 mg). The median cumulative doses in the intervention and non-intervention groups were 32200 mg and 17400 mg, respectively, showing a significant difference ($P = 0.047$). The median daily dose was 480 mg (range: 392-800 mg). The administration of sorafenib was discontinued in 38 patients for the following reasons: a reduction in the effects of administration in 16 (42%); grade 3 or higher AEs in 14 (36%); exacerbation of the general condition in 4 (11%); and other factors in 4 (11%). The mean medication possession ratios (MPRs) in the intervention and non-intervention groups were 97% and 85%, respectively, showing a significant difference ($P < 0.001$).

The results of the univariate- and multivariate analyses regarding the TTF are presented in Table 4. The multivariate analysis showed that significant factors for TTF prolongation included intervention by pharmacists (HR = 0.425, 95%CI: 0.186-0.971; $P = 0.042$) and MKI-associated HFSR (HR = 0.418, 95%CI: 0.175-0.994; $P = 0.048$). The median TTFs were 145 d (4.8 mo) in the intervention group and 43 d (1.4 mo) in the non-intervention group.

DISCUSSION

Sorafenib treatment is performed worldwide as evidence-based chemotherapy for advanced hepatocellular carcinoma[1-4]. A previous study reported that the rate of decreased in AFP levels early after the start of treatment was useful as a surrogate marker for sorafenib therapy[20]. A number of studies have indicated that a relationship exists between AEs after the start of sorafenib administration and antitumor effects; however, this relationship has not yet been confirmed. An international multicenter cooperative study showed by multivariate analysis that diarrhea, hypertension, and HFSR were

significant, independent factors associated with OS[21]. In addition, a meta-analysis of the data obtained from 1017 subjects, involving 12 cohort studies, showed that HFSR was a beneficial indicator that improves the prognosis[22].

In the present study, the multivariate analysis of OS showed that MKI-associated HFSR and BCLC B were significant predictive factors for the prolongation of OS. A previous study also indicated that OS was significantly prolonged in the BCLC B group (8.4 mo in BCLC C *vs* 20.6 mo in BCLC B patients ($P < 0.0001$))[23]. In the present study, all patients previously underwent TACE, except for one patient, in the BCLC B group (15/16: 94%). Although the introduction of sorafenib for non-responders to TACE may be a good option, the timing of its introduction has not yet been established. Furthermore, a study reported that the combination of sorafenib and TACE improved the TTP[24]. We did not combine sorafenib therapy with other treatments, such as TACE; a surrogate marker for an improvement in OS was obtained by monotherapy with sorafenib. We established a double-check system consisting of primary outpatient care by pharmacists specializing in cancer treatment and secondary outpatient care by physicians. The findings indicated that MKI-associated HFSR was a surrogate marker for OS.

Regarding the TTF, the multivariate analysis showed that intervention by pharmacists and MKI-associated HFSR significantly extended the treatment duration. This finding is consistent with the findings of a previous study indicating that intervention by nurses improved the efficacy of sorafenib treatment[13].

Sorafenib is an MKI with a high incidence of AEs. Primary AEs include HFSR, diarrhea, hypertension, nausea, and hemorrhage. MKI-associated HFSR occurs in approximately 1/3 of patients[25,26]. Furthermore, MKI-associated HFSR differs from previously reported HFSR induced by chemotherapy (*e.g.*, capecitabine, 5-fluorouracil, or doxorubicin)[27,28]. The time to onset of MKI-associated HFSR (14-28 d) is much shorter than that of HFSR induced by chemotherapy, and the site is localized (*e.g.*, areas in contact with shoes). MKI-associated cornification is also prominent. Furthermore, there is no evidence-based rationale to advocate for any of preventive measures for HFSR induced by chemotherapy for the prophylaxis of MKI-associated HFSR[29].

Patients receiving anticancer therapies have been considered to achieve a higher adherence rate than those with other chronic diseases. However, many studies have indicated that the adherence rate is not 100%, even in cancer patients receiving anticancer drugs[30-32]. Concerning the adherence of cancer patients to their treatment with oral anticancer drugs, a previous study investigated breast cancer patients receiving oral anticancer drugs and reported that treatment-associated toxicities affected effective cancer treatments, suggesting the usefulness of adequate interventions for increasing adherence[33]. Furthermore, another study indicated that long-term therapy with oral anticancer drugs reduced the risk of death, while a low adherence rate increased the

risk of death[34]. In addition, a decrease in the adherence rates was shown to reduce treatment responses in imatinib-treated patients with chronic myeloid leukemia[32,35].

Insufficient adherence may hinder the effects of oral anticancer drugs. If physicians responsible for prescriptions are unaware of poor adherence, they may change the regimen unnecessarily based on a misunderstanding of disease progression related to insufficient drug effects. Factors associated with non-adherence for a recommended treatment regimen include individual patient characteristics, the features of the disease and treatment regimen, and aspects of the medical care system[36]. Individual factors form a system by influencing each other, and if a patient's disease is not regarded as a system abnormality, effective treatment may not be achieved. The time to onset of MKI-associated HFSR is much shorter than for that of HFSR induced by chemotherapy. Moreover, there is no evidence-based rationale to advocate for preventive measures for MKI-associated HFSR. Sorafenib treatment is less effective for tumor shrinkage; however, intervention by healthcare providers, including pharmacists with special knowledge, can cover these individual factors. In the present study, the median cumulative doses in the intervention and the non-intervention groups were 32200 mg and 17400 mg, respectively, showing a significant difference (P = 0.047). The MPR was significantly greater in the intervention group than and the non-intervention group, at 97% and 85% respectively, showing a significant difference (P < 0.001); intervention by pharmacists contributed to this increased adherence.

To evaluate the role of MKI-associated HFSR as a surrogate marker for therapeutic effects, we conducted a retrospective analysis involving advanced hepatocellular carcinoma patients who had received sorafenib treatment under intervention by pharmacists. The results indicated that MKI-associated HFSR was a surrogate marker for OS under increased adherence. Neither the rate of decrease in AFP levels in the early stage nor diarrhea/hypertension was a surrogate marker. Intervention by pharmacists prolonged the TTF and increased the median cumulative dose and the MPR, resulting in better adherence. A borderline significant difference was observed in terms of OS between patients with and without intervention by pharmacists. The present study had the following limitations: our data are based on a retrospective study, and the sample size was small.

In conclusion, intervention by pharmacists using the double-check system improved drug adherence. Under increased adherence, MKI-associated HFSR in patients receiving sorafenib treatment was a surrogate marker reflecting a better prognosis. Intervention by healthcare providers, including pharmacists with special knowledge, needs to be performed for adequate sorafenib treatment.

ARTICLE HIGHLIGHTS

Research background
A surrogate marker that reflects a better prognosis after the introduction of sorafenib has not yet been established.

Research motivation
The incidence of adverse events after sorafenib introduction is high, reducing drug adherence.

Research objectives
The authors performed intervention by pharmacists using the double-check system to improve drug adherence. In addition, they evaluated a surrogate marker reflecting a better prognosis after sorafenib introduction under increased adherence.

Research methods
This retrospective study was conducted on advanced hepatocellular carcinoma patients who had been treated with sorafenib between May 2009 and March 2017. Establishing the overall survival (OS) as a primary endpoint, the authors evaluated a surrogate marker using multivariate analysis. Furthermore, the effects of intervention by pharmacists on drug adherence were assessed using the time to treatment failure (TTF), median cumulative dose, and medication possession ratio (MPR).

Research results
The subjects were 40 patients who had undergone sorafenib therapy. In the presence of grade 1 or higher multikinase inhibitor (MKI)-associated hand-foot skin reaction (HFSR), OS was significantly prolonged. The results of multivariate analysis showed that the presence of MKI-associated HFSR (hazard ratio = 0.241, 95%CI: 0.102-0.567; P = 0.001) was one of the significant predictive factors for the prolongation of OS. There were significant differences in the TTF, median cumulative dose, and MPR between the intervention and non-intervention groups.

Research conclusions
Intervention by pharmacists increased the drug adherence. The MKI-associated HFSR was an advantageous surrogate marker under increased adherence. Intervention by healthcare providers needs to be performed for adequate sorafenib treatment.

Research perspectives
It remains to be clarified whether intervention by healthcare providers improves the prognosis of hepatocellular carcinoma patients after sorafenib introduction. Therefore, the double-check system should be further improved, and a larger number of patients should be evaluated. Furthermore, a prospective study should be conducted.

ACKNOWLEDGMENTS

REFERENCES

1   **Bruix J**, Raoul JL, Sherman M, Mazzaferro V, Bolondi L, Craxi A, Galle PR, Santoro A, Beaugrand M, Sangiovanni A, Porta C, Gerken G, Marrero JA, Nadel A, Shan M, Moscovici M, Voliotis D, Llovet JM. Efficacy and safety of sorafenib in patients with advanced hepatocellular carcinoma: subanalyses of a phase III trial. *J Hepatol* 2012; **57**: 821-829 [PMID: 22727733 DOI: 10.1016/j.jhep.2012.06.014]
2   **Llovet JM**, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J; SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378-390 [PMID: 18650514 DOI: 10.1056/NEJMoa0708857]
3   **Cheng AL**, Guan Z, Chen Z, Tsao CJ, Qin S, Kim JS, Yang TS, Tak WY, Pan H, Yu S, Xu J, Fang F, Zou J, Lentini G, Voliotis

D, Kang YK. Efficacy and safety of sorafenib in patients with advanced hepatocellular carcinoma according to baseline status: subset analyses of the phase III Sorafenib Asia-Pacific trial. *Eur J Cancer* 2012; **48**: 1452-1465 [PMID: 22240282 DOI: 10.1016/j.ejca.2011.12.006]

4   **Cheng AL**, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, Luo R, Feng J, Ye S, Yang TS, Xu J, Sun Y, Liang H, Liu J, Wang J, Tak WY, Pan H, Burock K, Zou J, Voliotis D, Guan Z. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 2009; **10**: 25-34 [PMID: 19095497 DOI: 10.1016/S1470-2045(08)70285-7]

5   **Cheng AL**, Kang YK, Lin DY, Park JW, Kudo M, Qin S, Chung HC, Song X, Xu J, Poggi G, Omata M, Pitman Lowenthal S, Lanzalone S, Yang L, Lechuga MJ, Raymond E. Sunitinib versus sorafenib in advanced hepatocellular cancer: results of a randomized phase III trial. *J Clin Oncol* 2013; **31**: 4067-4075 [PMID: 24081937 DOI: 10.1200/JCO.2012.45.8372]

6   **Johnson PJ**, Qin S, Park JW, Poon RT, Raoul JL, Philip PA, Hsu CH, Hu TH, Heo J, Xu J, Lu L, Chao Y, Boucher E, Han KH, Paik SW, Robles-Aviña J, Kudo M, Yan L, Sobhonslidsuk A, Komov D, Decaens T, Tak WY, Jeng LB, Liu D, Ezzeddine R, Walters I, Cheng AL. Brivanib versus sorafenib as first-line therapy in patients with unresectable, advanced hepatocellular carcinoma: results from the randomized phase III BRISK-FL study. *J Clin Oncol* 2013; **31**: 3517-3524 [PMID: 23980084 DOI: 10.1200/JCO.2012.48.4410]

7   **Bruix J**, Qin S, Merle P, Granito A, Huang YH, Bodoky G, Pracht M, Yokosuka O, Rosmorduc O, Breder V, Gerolami R, Masi G, Ross PJ, Song T, Bronowicki JP, Ollivier-Hourmand I, Kudo M, Cheng AL, Llovet JM, Finn RS, LeBerre MA, Baumhauer A, Meinhardt G, Han G; RESORCE Investigators. Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet* 2017; **389**: 56-66 [PMID: 27932229 DOI: 10.1016/S0140-6736(16)32453-9]

8   **Llovet JM**, Peña CE, Lathia CD, Shan M, Meinhardt G, Bruix J; SHARP Investigators Study Group. Plasma biomarkers as predictors of outcome in patients with advanced hepatocellular carcinoma. *Clin Cancer Res* 2012; **18**: 2290-2300 [PMID: 22374331 DOI: 10.1158/1078-0432.CCR-11-2175]

9   **Vincenzi B**, Santini D, Russo A, Addeo R, Giuliani F, Montella L, Rizzo S, Venditti O, Frezza AM, Caraglia M, Colucci G, Del Prete S, Tonini G. Early skin toxicity as a predictive factor for tumor control in hepatocellular carcinoma patients treated with sorafenib. *Oncologist* 2010; **15**: 85-92 [PMID: 20051477 DOI: 10.1634/theoncologist.2009-0143]

10 **Otsuka T**, Eguchi Y, Kawazoe S, Yanagita K, Ario K, Kitahara K, Kawasoe H, Kato H, Mizuta T; Saga Liver Cancer Study Group. Skin toxicities and survival in advanced hepatocellular carcinoma patients treated with sorafenib. *Hepatol Res* 2012; **42**: 879-886 [PMID: 22469363 DOI: 10.1111/j.1872-034X.2012.00991.x]

11 **Di Costanzo GG**, Tortora R, Iodice L, Lanza AG, Lampasi F, Tartaglione MT, Picciotto FP, Mattera S, De Luca M. Safety and effectiveness of sorafenib in patients with hepatocellular carcinoma in clinical practice. *Dig Liver Dis* 2012; **44**: 788-792 [PMID: 22579445 DOI: 10.1016/j.dld.2012.04.001]

12 **Reig M**, Torres F, Rodriguez-Lope C, Forner A, LLarch N, Rimola J, Darnell A, Ríos J, Ayuso C, Bruix J. Early dermatologic adverse events predict better outcome in HCC patients treated with sorafenib. *J Hepatol* 2014; **61**: 318-324 [PMID: 24703956 DOI: 10.1016/j.jhep.2014.03.030]

13 **Shomura M**, Kagawa T, Shiraishi K, Hirose S, Arase Y, Koizumi J, Mine T. Skin toxicity predicts efficacy to sorafenib in patients with advanced hepatocellular carcinoma. *World J Hepatol* 2014; **6**: 670-676 [PMID: 25276283 DOI: 10.4254/wjh.v6.i9.670]

14 **Bruix J**, Sherman M; American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53**: 1020-1022 [PMID: 21374666 DOI: 10.1002/hep.24199]

15 **Forner A**, Llovet JM, Bruix J. Hepatocellular carcinoma. *Lancet* 2012; **379**: 1245-1255 [PMID: 22353262 DOI: 10.1016/S0140-6736(11)61347-0]

16 **Lencioni R**, Llovet JM. Modified RECIST (mRECIST) assessment for hepatocellular carcinoma. *Semin Liver Dis* 2010; **30**: 52-60 [PMID: 20175033 DOI: 10.1055/s-0030-1247132]

17 **Peto R**, Pike MC, Armitage P, Breslow NE, Cox DR, Howard SV, Mantel N, McPherson K, Peto J, Smith PG. Design and analysis of randomized clinical trials requiring prolonged observation of each patient. II. analysis and examples. *Br J Cancer* 1977; **35**: 1-39 [PMID: 831755 DOI: 10.1038/bjc.1977.1]

18 **Cox DR**. Regression models and life tables. *J R Stat Soc B* 1972; **34**: 187-220

19 **Anderson JR**, Cain KC, Gelber RD. Analysis of survival by tumor response. *J Clin Oncol* 1983; **1**: 710-719 [PMID: 6668489 DOI: 10.1200/JCO.1983.1.11.710]

20 **Personeni N**, Bozzarelli S, Pressiani T, Rimassa L, Tronconi MC, Sclafani F, Carnaghi C, Pedicini V, Giordano L, Santoro A. Usefulness of alpha-fetoprotein response in patients treated with sorafenib for advanced hepatocellular carcinoma. *J Hepatol* 2012; **57**: 101-107 [PMID: 22414760 DOI: 10.1016/j.jhep.2012.02.016]

21 **Howell J**, Pinato DJ, Ramaswami R, Bettinger D, Arizumi T, Ferrari C, Yen C, Gibbin A, Burlone ME, Guaschino G, Sellers L, Black J, Pirisi M, Kudo M, Thimme R, Park JW, Sharma R. On-target sorafenib toxicity predicts improved survival in hepatocellular carcinoma: a multi-centre, prospective study. *Aliment Pharmacol Ther* 2017; **45**: 1146-1155 [PMID: 28252185 DOI: 10.1111/apt.13977]

22 **Wang P**, Tan G, Zhu M, Li W, Zhai B, Sun X. Hand-foot skin reaction is a beneficial indicator of sorafenib therapy for patients with hepatocellular carcinoma: a systemic review and meta-analysis. *Expert Rev Gastroenterol Hepatol* 2018; **12**: 1-8 [PMID: 28847184 DOI: 10.1080/17474124.2017.1373018]

23 **Iavarone M**, Cabibbo G, Piscaglia F, Zavaglia C, Grieco A, Villa E, Cammà C, Colombo M; SOFIA (SOraFenib Italian Assessment) study group. Field-practice study of sorafenib therapy for hepatocellular carcinoma: a prospective multicenter study in Italy. *Hepatology* 2011; **54**: 2055-2063 [PMID: 21898496 DOI: 10.1002/hep.24644]

24 **Li J**, Liu W, Zhu W, Wu Y, Wu B. Transcatheter hepatic arterial chemoembolization and sorafenib for hepatocellular carcinoma: a meta-analysis of randomized, double-blind controlled trials. *Oncotarget* 2017; **8**: 59601-59608 [PMID: 28938663 DOI: 10.18632/oncotarget.19334]

25 **Chu D**, Lacouture ME, Fillos T, Wu S. Risk of hand-foot skin reaction with sorafenib: a systematic review and meta-analysis. *Acta Oncol* 2008; **47**: 176-186 [PMID: 18210295 DOI: 10.1080/02841860701765675]

26 **Lacouture ME**, Wu S, Robert C, Atkins MB, Kong HH, Guitart J, Garbe C, Hauschild A, Puzanov I, Alexandrescu DT, Anderson RT, Wood L, Dutcher JP. Evolving strategies for the management of hand-foot skin reaction associated with the multitargeted kinase inhibitors sorafenib and sunitinib. *Oncologist* 2008; **13**: 1001-1011 [PMID: 18779536 DOI: 10.1634/theoncologist.2008-0131]

27 **Autier J**, Mateus C, Wechsler J, Spatz A, Robert C. [Cutaneous side effects of sorafenib and sunitinib]. *Ann Dermatol Venereol* 2008; **135**: 148-153; quiz 147, 154 [PMID: 18342102 DOI: 10.1016/j.annder.2007.12.006]

28 **Heidary N**, Naik H, Burgin S. Chemotherapeutic agents and the skin: An update. *J Am Acad Dermatol* 2008; **58**: 545-570 [PMID: 18342708 DOI: 10.1016/j.jaad.2008.01.001]

29 **Anderson R**, Jatoi A, Robert C, Wood LS, Keating KN, Lacouture ME. Search for evidence-based approaches for the prevention and palliation of hand-foot skin reaction (HFSR) caused by the multikinase inhibitors (MKIs). *Oncologist* 2009; **14**: 291-302 [PMID: 19276294 DOI: 10.1634/theoncologist.2008-0237]

30 **Partridge AH**, Archer L, Kornblith AB, Gralow J, Grenier D, Perez E, Wolff AC, Wang X, Kastrissios H, Berry D, Hudis C, Winer E, Muss H. Adherence and persistence with oral adjuvant chemotherapy in older women with early-stage breast cancer in CALGB 49907: adherence companion study 60104. *J Clin Oncol* 2010; **28**: 2418-2422 [PMID: 20368559 DOI: 10.1200/JCO.2009.26.4671]

31   **Noens L**, van Lierde MA, De Bock R, Verhoef G, Zachée P, Berneman Z, Martiat P, Mineur P, Van Eygen K, MacDonald K, De Geest S, Albrecht T, Abraham I. Prevalence, determinants, and outcomes of nonadherence to imatinib therapy in patients with chronic myeloid leukemia: the ADAGIO study. *Blood* 2009; **113**: 5401-5411 [PMID: 19349618 DOI: 10.1182/blood-2008-12-196543]

32   **Marin D**, Bazeos A, Mahon FX, Eliasson L, Milojkovic D, Bua M, Apperley JF, Szydlo R, Desai R, Kozlowski K, Paliompeis C, Latham V, Foroni L, Molimard M, Reid A, Rezvani K, de Lavallade H, Guallar C, Goldman J, Khorashad JS. Adherence is the critical factor for achieving molecular responses in patients with chronic myeloid leukemia who achieve complete cytogenetic responses on imatinib. *J Clin Oncol* 2010; **28**: 2381-2388 [PMID: 20385986 DOI: 10.1200/JCO.2009.26.3087]

33   **Hershman DL**, Kushi LH, Shao T, Buono D, Kershenbaum A, Tsai WY, Fehrenbacher L, Gomez SL, Miles S, Neugut AI. Early discontinuation and nonadherence to adjuvant hormonal therapy in a cohort of 8,769 early-stage breast cancer patients. *J Clin Oncol* 2010; **28**: 4120-4128 [PMID: 20585090 DOI: 10.1200/JCO.2009.25.9655]

34   **McCowan C**, Shearer J, Donnan PT, Dewar JA, Crilly M, Thompson AM, Fahey TP. Cohort study examining tamoxifen adherence and its relationship to mortality in women with breast cancer. *Br J Cancer* 2008; **99**: 1763-1768 [PMID: 18985046 DOI: 10.1038/sj.bjc.6604758]

35   **Ibrahim AR**, Eliasson L, Apperley JF, Milojkovic D, Bua M, Szydlo R, Mahon FX, Kozlowski K, Paliompeis C, Foroni L, Khorashad JS, Bazeos A, Molimard M, Reid A, Rezvani K, Gerrard G, Goldman J, Marin D. Poor adherence is the main reason for loss of CCyR and imatinib failure for chronic myeloid leukemia patients on long-term therapy. *Blood* 2011; **117**: 3733-3736 [PMID: 21346253 DOI: 10.1182/blood-2010-10-309807]

36   **Ruddy K**, Mayer E, Partridge A. Patient adherence and persistence with oral anticancer treatment. *CA Cancer J Clin* 2009; **59**: 56-66 [PMID: 19147869 DOI: 10.3322/caac.20004]

ORIGINAL ARTICLE

Observational Study

Health behaviors of Korean adults with hepatitis B: Findings of the 2016 Korean National Health and Nutrition Examination Survey

Yu-Hyeon Yi, Yun-Jin Kim, Sang-Yeoup Lee, Byung-Mann Cho, Young-Hye Cho, Jeong-Gyu Lee

Yu-Hyeon Yi, Yun-Jin Kim, Young-Hye Cho, Jeong-Gyu Lee, Department of Family Medicine, Pusan National University School of Medicine, Yangsan-si 50612, South Korea

Yu-Hyeon Yi, Yun-Jin Kim, Byung-Mann Cho, Jeong-Gyu Lee, Medical Research Institute, Pusan National University Hospital, Busan 49241, South Korea

Yu-Hyeon Yi, Jeong-Gyu Lee, Busan Tobacco Control Center, Pusan National University Hospital, Busan 49241, South Korea

Sang-Yeoup Lee, Department of Medical Education, Pusan National University School of Medicine, Yangsan-si 50612, South Korea

Sang-Yeoup Lee, Young-Hye Cho, Family Medicine Clinic, Pusan National University Yangsan Hospital, Yangsan-si 50612, South Korea

Byung-Mann Cho, Department of Preventive Medicine and Occupational Medicine, Pusan National University School of Medicine, Yangsan-si 50612, South Korea

ORCID number: Yu-Hyeon Yi (0000-0002-1786-2737); Yun-Jin Kim (0000-0002-0204-3253); Sangyeoup Lee (0000-0002-3585-9910); Byung-Mann Cho (0000-0003-0893-3617); Young-Hye Cho (0000-0003-2176-6227); Jeong-Gyu Lee (0000-0001-7160-0714).

Author contributions: Yi YH, Kim YJ, Lee SY, Cho BM, Cho YH, Lee JG contributed to study conception and design; Yi YH and Lee JG contributed to data analysis and interpretation, and drafting of manuscript; Lee SY and Cho BM contributed data management; Yi YH, Kim YJ, Lee SY, Cho BM, Cho YH, Lee JG contributed to reviewing and final approval of article; Lee JG contributed to revising manuscript and finalizing submission.

Institutional review board statement: Our study design was approved by the Institutional Review Board of Pusan National University Hospital (approval ID: H-1805-017-067)

Conflict-of-interest statement: All authors have no conflict of interest to disclose.

STROBE statement: The authors have read the STROBE Statement checklist of items, and the manuscript was prepared and revised according to the STROBE Statement checklist of items.

Correspondence to: Jeong-Gyu Lee, MD, PhD, Associate Professor, Department of Family Medicine, Pusan National University School of Medicine, Yangsan-si 50612, South Korea. jeklee@pnu.edu
Telephone: +82-51-2407834
Fax: +82-51-2407843

Abstract

AIM
To assess the frequencies of five health-related behaviors (smoking, alcohol consumption, body weight, sleep dura-

tion, and physical activity) in Korean adults with chronic hepatitis B.

METHODS

Data were obtained from the 2016 Korean National Health and Nutrition Examination Survey. In total, 5887 subjects (2568 males, 3319 females) over 19 years old were enrolled in this study. Interviews were performed to obtain information on demographic characteristics and medical conditions. A self-administered questionnaire and medical examination were used to assess the smoking history, alcohol use, physical activity, sleep duration, and body weight of the subjects. Chronic hepatitis B was diagnosed based on detection of hepatitis B surface antigen (HBsAg). The subjects were categorized into HBsAg positive and negative groups, and a complex sampling analysis was conducted to compare the health behaviors between these groups.

RESULTS

Among males, the current smoking rate in the HBsAg positive group was higher than that in the negative group (45.5% *vs* 38.5%). In the positive group, the rates of monthly and high-risk alcohol use were 70.4% and 17.6% in males and 45.9% and 3.8% in females, respectively. The rate of alcohol use was similar between the two groups [$P = 0.455$ (males) and $P = 0.476$ (females)]. In the HBsAg positive group, 32.3% and 49.9% of males and 26.5% and 49.6% of females were overweight and physically inactive, respectively. High-risk alcohol consumption and physical inactivity were significantly associated with self-perceived health status.

CONCLUSION

Our data demonstrate that a large proportion of Korean adults with chronic hepatitis B have poor health behaviors. Further studies are needed to confirm our results.

Key words: Health behavior; Self-perceived health status; Hepatitis; Health risk behavior; Health status

Core tip: A large proportion of Korean adults with chronic hepatitis B have poor health behaviors, particularly in terms of smoking and alcohol consumption. High-risk alcohol consumption and physical inactivity are significantly associated with self-perceived health status. Because it is a risk factor for hepatocellular carcinoma, individuals with chronic hepatitis B should maintain a healthy lifestyle. They should be encouraged to improve their health behaviors and participate in appropriate education programs.

INTRODUCTION

Hepatitis B is an important public health issue in the Asia-Pacific region. It is estimated that 240 million people are infected with hepatitis B virus (HBV)[1]. Chronic HBV infection may cause premature death from uncompensated liver cirrhosis and hepatocellular carcinoma (HCC). Primary liver cancer is the second leading cause of cancer-related mortality[2]. Chronic HBV infection, cirrhosis, and hepatitis C viral infection are established risk factors for HCC[2]. The modifiable risk factors for HCC include lifetime alcohol consumption[3,4], tobacco smoking[5], obesity[6], diabetes[6], nonalcoholic fatty liver disease[7], and socioeconomic status[3]. Indeed, lifetime moderate alcohol use may cause HCC in older people, and diabetes may increase the risk of HCC independently of cirrhosis[8,9].

Health-related behaviors such as tobacco smoking, alcohol consumption, body weight status, sleep duration, and physical activity are important for the management of chronic medical conditions[10,11]. Smoking, physical activity, and alcohol consumption are the most critical behavioral determinants of health[12]. Furthermore, health behaviors are important following the onset of disease, because they can reduce disease severity and risk of recurrence and increase survival duration[12]. Modification of health behaviors is a particularly important intervention for patients with chronic hepatitis B.

High-quality chronic-care systems that include preventive management of health behaviors are needed to improve the quality of life of patients with chronic conditions[13]. Subjective awareness of health status can be used to evaluate one's own health and the prognosis of various chronic diseases[14]. To our knowledge, little is known about the relationships between health behaviors and subjective health status.

In this study, we evaluated the frequencies of five health-related behaviors (smoking, alcohol consumption, body weight, sleep duration, and physical activity) in Korean adults with hepatitis B. We also examined the association between these health behaviors and self-perceived health status.

MATERIALS AND METHODS

Data resources

Data were obtained from the 2016 Korean National Health and Nutrition Examination Survey (KNHANES). The KNHANES is a nationwide representative cross-sectional survey conducted by the Korea Center for Disease Control and Prevention (KCDC) beginning in 1998. The KNHANES is an ongoing survey that assesses the health status of Koreans and monitors the trends in health risk factors and the prevalence of major chronic diseases in Korea[15]. The KNHANES comprises a

Table 1 Demographic and clinical characteristics of the participants in the 2016 Korean National Health and Nutrition Examination Survey (*n* = 5887)

Variable	Sample size	Mean/ proportion	95%CI
Demographics			
Age (yr)	5887	46.7	46.0-47.5
Male (%)	2568	50.3	49.1-50.5
Low income level (%)	1129	16.2	13.7-18.4
Presence of spouse (%)	4970	77.3	75.4-79.1
Education ≥ 10 yr (%)	3839	75.8	71.3-80.3
Current medical history			
Hypertension	1387	18.9	17.6-20.4
Diabetes mellitus	575	7.8	7.0-8.7
Dyslipidemia	799	11.3	10.3-12.4
Depression	166	2.6	2.1-3.1
Liver cirrhosis	12	0.2	0.1-0.3
Hepatitis B	192	3.4	2.9-4.0
Anthropometric measurements			
BMI (kg/m^2)		24.0	23.9-24.1
Abdominal circumference (cm)		82.9	82.4-83.3
Systolic BP (mm Hg)		117.9	117.3-118.5
AST (IU/L)		22.7	22.2-23.2
ALT (IU/L)		22.7	22.2-23.3
Fasting plasma glucose (mg/dL)		100.4	99.6-101.3
HbA1c (%)		5.6	5.6-5.7
Total cholesterol (mg/dL)		193.1	191.9-194.4
Triglycerides (mg/dL)		144.8	139.3-150.4
LDL-cholesterol (mg/dL)		117.4	114.9-119.8
hs-CRP (mg/L)		1.23	1.17-1.29

BMI: Body mass index; BP: Blood pressure; AST: Aspartate transferase; ALT: Alanine transferase; HbA1c: Hemoglobin A1c; LDL: Low-density lipoprotein; hs-CRP: High-sensitivity C reactive protein.

complex, stratified, multistage sample in which household units are selected according to geographic area, sex, and age group. Informed consent was obtained from all of the subjects. The 2016 KNHANES response rate was 72.2% for the interview and health examination. This study involved 5887 subjects (2568 males, 3319 females) over 19 years of age.

Measures
The interview and health examinations were performed by a trained interviewer and medical staff using calibrated equipment according to a standardized protocol. The interviewer collected demographic characteristics including housing characteristics, medical conditions, socioeconomic status, and education levels. The self-administered questionnaire included questions on smoking status, alcohol use, physical activity, sleep health, and weight control. The health examination was conducted in a mobile examination center and consisted of a physical examination, anthropometric measurements, and blood and urine laboratory tests. Height and weight were measured to the nearest 0.1 cm and 0.1 kg, respectively, using a standard protocol. Body mass index (BMI) was calculated as body weight (kg) divided by height in meters squared (m^2). Waist circumference was measured at the end of a normal expiration of breath and to the nearest 0.1 cm. Blood pressure was measured using an automated blood pressure measurement device. Blood

and urine samples were subsequently analyzed at a certified laboratory. The performance of this laboratory analysis was monitored by a laboratory data quality-control program to ensure that the data met the accuracy standard[15]. Hepatitis B surface antigen (HBsAg) levels were measured using Elecsys HBsAgⅡ (Roche/Germany) electrochemiluminescence immunoassay (ELICA). Subjects were classified as HBsAg positive or negative (hereafter, the negative and positive groups) according to their serology results.

Definitions of health behaviors
Current cigarette smoking was defined as smoking at least 100 cigarettes over a lifetime and currently smoking cigarettes everyday (daily) or on some days (nondaily)[16]. No alcohol consumption was defined as lifetime abstention and consumption of < 1 drink per month for the past year. Monthly alcohol use was defined as consumption of ≥ 1 alcoholic drink per month for the past year. High-risk alcohol use was defined as consumption of ≥ 7 (males) or ≥ 5 (females) alcoholic drinks more than twice a week[17]. Body weight was classified as underweight (BMI < 18.5 kg/m^2), normal weight (BMI: 18.5-25.0 kg/m^2), or overweight (BMI > 25.0 kg/m^2)[18]. Adequate sleep was defined as self-reported ≥ 7 h per night of sleep[19]. Sufficient physical activity was defined as performance of moderate-to-vigorous physical activity (MVPA) for ≥ 150 min per week[20].

Self-perceived health status
The subjective health status of the subjects was evaluated using a Likert-scale-based questionnaire. The question "How is your health in general?" was used to assess self-perceived health status, and the possible responses ranged from 1 (very good) to 5 (very poor).

Statistical analysis
Data were subjected to a weighted complex sampling analysis. Demographic and clinical characteristics are shown as numbers, means, and 95% confidence intervals (95%CI). A chi-squared test was performed for comparisons of health behaviors between the two groups. A *t*-test using a general linear model was used to compare self-perceived health status between the two groups. A multivariate linear regression analysis was performed to examine the associations between the health behaviors and self-perceived health status. A value of *P* < 0.05 was considered indicative of statistical significance. Statistical analyses were performed using IBM® SPSS® Statistics, version 23.0, for Windows™.

RESULTS

Demographic and clinical characteristics of the subjects
This study involved 5887 subjects, of whom 2568 (50.3%) were males and 3319 (49.7%) were females. Table 1 shows the demographic and clinical characteristics of the subjects. The mean age of the subjects was 46.7

Table 2 Health behaviors and self-perceived health status in Korean adult males according to hepatitis B surface antigen status (n = 2568)

Variable	Total (n = 2568)	HBsAg negative (n = 2481)	HBsAg positive (n = 87)	P value[1]
	Mean ± SE	Mean ± SE	Mean ± SE	
Health behavior				
Current cigarette smoker	38.6 ± 1.3	38.5 ± 1.3	41.5 ± 5.7	0.591
Alcohol use				0.455
Non-drinker	25.2 ± 1.1	25.1 ± 1.1	29.6 ± 5.9	
Monthly	74.8 ± 1.1	74.9 ± 1.1	70.4 ± 5.9	
High risk	21.1 ± 1.0	21.3 ± 1.0	17.6 ± 4.5	
Body weight status				0.071
Underweight	2.6 ± 0.4	2.7 ± 0.4	0.8 ± 0.8	
Normal	55.5 ± 1.2	55.5 ± 1.2	66.9 ± 5.4	
Overweight	41.9 ± 1.3	42.2 ± 1.3	32.3 ± 5.4	
Sleep duration				0.962
Short	37.9 ± 1.2	37.9 ± 1.2	37.6 ± 5.9	
Adequate	62.1 ± 1.2	62.1 ± 1.2	62.4 ± 5.9	
MVPA				0.840
Inactive	48.6 ± 1.4	48.6 ± 1.4	49.9 ± 6.4	
Sufficiently active	51.4 ± 1.4	51.4 ± 1.4	50.1 ± 6.4	
Self-perceived health status (range 1-5)	3.12 ± 0.04	3.13 ± 0.04	2.97 ± 0.10	0.146

[1]Chi-squared test, except for self-perceived health status (general linear model by complex sampling analysis). Current cigarette smoker: Smoked at least 100 cigarettes in their lifetime and currently smoked cigarettes every day (daily) or on some days (nondaily). Monthly alcohol use: Consumption of more than 1 drink per month in the past year. Non-drinker: Lifetime abstainer and subjects who drank less than 1 drink per month in the past year. High-risk alcohol use: Consumption of at least 7 (males) or 5 (females) alcoholic drinks more than two times per week. Adequate sleep: ≥ 7 h per night of self-reported sleep. MVPA: Sufficiently active was defined as ≥ 150 min/wk of moderate-to-vigorous physical activity. HBsAg: Hepatitis B surface antigen.

years (95%CI: 46.0-47.5 years), and 3839 (75.8%) had ≥ 10 years of education. The chronic medical conditions were hypertension (n = 1387; 18.9%, 95%CI: 17.6%-20.4%), diabetes (n = 575; 7.8% 95%CI: 7.0%-8.7%), dyslipidemia (n = 799; 11.3%, 95%CI: 10.3%-12.4%), depression (n = 166; 2.6%, 95%CI: 2.1%-3.1%), liver cirrhosis (n = 12; 0.2%, 95%CI: 0.1%-0.3%), and hepatitis B (n = 192; 3.4%, 95%CI: 2.9%-4.0%), respectively. The mean BMI and abdominal circumference of the subjects were 24.0 (95%CI: 23.9-24.1) kg/m^2 and 82.9 (95%CI: 82.4-83.3) cm, respectively. The fasting plasma glucose and hemoglobin A1c (HbA1c) levels were 100.4 (95%CI: 99.6-101.3) mg/dL and 5.6% (95%CI: 5.6%-5.7%), respectively. The total cholesterol and low-density lipoprotein (LDL)-cholesterol levels of the subjects were 193.1 (95%CI: 191.9-194.4) and 117.4 (95%CI: 114.9-119.8) mg/dL, respectively. The mean high-sensitivity C-reactive protein (hs-CRP) level was 1.23 (95%CI: 1.17-1.29) mg/L.

Health behaviors and self-perceived health status
The health behaviors and self-perceived health status of the subjects are shown in Tables 2 and 3. The current cigarette smoking rate in males was non-significantly higher in the positive group than the negative group (41.5% *vs* 38.5%; P = 0.591). In the positive group, the rates of monthly and high-risk alcohol use were 70.4% and 17.6% in males and 45.9% and 3.8% in females, respectively [P = 0.455 (males) and P = 0.476 (females)]. More males had a normal body weight in the positive group than the negative group (P = 0.071). More females reported an adequate sleep duration in the

negative group than the positive group (P = 0.452). The rate of sufficient physical activity was similar between the positive and negative groups among males but higher in the positive group than the negative group among females (P = 0.288). The self-perceived health status was similar between the positive and negative groups among males and females.

Associations between health behaviors and self-perceived health status
High-risk alcohol consumption (β = -0.605, P = 0.020) and physical inactivity (β = 0.348, P = 0.013) were significantly associated with self-perceived health status in the positive group (Table 4). Current smoking (P = 0.078), body weight (P = 0.410), and inadequate sleep (P = 0.315) were not associated with self-perceived health status. LDL-cholesterol (P = 0.017) and hs-CRP (P = 0.001) levels were significantly associated with self-perceived health status after adjusting for age, systolic blood pressure, waist circumference, and HbA1c level.

DISCUSSION

In the present study, the rates of five health-related behaviors did not differ according to HBsAg positivity. Among males, current smoking rates were higher in the positive group, but there were no significant differences in alcohol use, body weight status, sleep duration, or physical activity between the two groups. In the positive group, the rates of high-risk alcohol use were 17.6% in males and 3.8% in females. High-risk alcohol consumption and physical inactivity were significantly

Table 3 Health behaviors and self-perceived health status in Korean adult females according to hepatitis B surface antigen status (*n* = 3319)

Variable	Total (*n* = 3319)	HBsAg negative (*n* = 3214)	HBsAg positive (*n* = 105)	*P* value[1]
	Mean ± SE	Mean ± SE	Mean ± SE	
Health behavior				
Current cigarette smoker	6.1 ± 0.6	6.2 ± 0.6	3.2 ± 1.9	0.260
Alcohol use				0.476
Non-drinker	54.9 ± 1.1	54.9 ± 1.1	54.1 ± 5.3	
Monthly	45.1 ± 1.1	45.1 ± 1.1	45.9 ± 5.3	
High risk	5.3 ± 0.5	5.3 ± 0.5	3.8 ± 1.8	
Body weight status				0.858
Underweight	5.6 ± 0.6	5.6 ± 0.6	5.5 ± 3.2	
Normal	65.1 ± 1.0	65.0 ± 1.0	68.0 ± 5.0	
Obese	29.3 ± 1.0	29.4 ± 1.1	26.5 ± 4.8	
Sleep duration				0.452
Short	38.1 ± 1.0	37.9 ± 1.2	37.6 ± 5.9	
Adequate	61.9 ± 1.0	62.0 ± 1.0	58.0 ± 5.3	
MVPA				0.288
Inactive	55.8 ± 1.2	56.0 ± 1.2	49.6 ± 6.0	
Sufficiently active	44.2 ± 1.2	44.0 ± 1.2	50.4 ± 6.0	
Self-perceived health status (range 1-5)	3.15 ± 0.04	3.15 ± 0.04	3.15 ± 0.14	0.992

[1]Chi-squared test (except for self-perceived health status: General linear model by complex sampling analysis). Current cigarette smoker: smoked at least 100 cigarettes in their lifetime and currently smoked cigarettes every day (daily) or on some days (nondaily). Monthly alcohol use: Consumption of > 1 drink per month in the past year. Non-drinker: lifetime abstainer and < 1 drink per month in the past year. High-risk alcohol use: Consumption of $\geq$ 7 (males) or $\geq$ 5 (females) alcoholic drinks more than twice a week. Adequate sleep: $\geq$ 7 h per night of self-reported sleep. MVPA: Sufficiently active was defined as $\geq$ 150 min/wk of moderate-to-vigorous physical activity. HBsAg: Hepatitis B surface antigen.

Table 4 Associations between health behaviors and self-perceived health status among Hepatitis B surface antigen positive adults in the 2016 Korean National Health and Nutrition Examination Survey

Variable	Self-perceived health status		*P* value[1]
	β	95%CI	
Health behavior			
Current cigarette smoker	0.569	-0.077, 1.215	0.078
Overweight	0.244	-0.388, 0.875	0.410
Sleeps less than 7 h	-0.281	-0.873, 0.311	0.315
High-risk alcohol use	-0.605	-1.092, -0.118	0.020
Physically inactive	0.348	0.089, 0.608	0.013
Covariates			
Age (yr)	0.003	-0.018, 0.024	0.778
SBP	0.015	-0.017, 0.047	0.331
WC	0.005	-0.067, 0.076	0.890
HbA1c	-0.279	-1.000, 0.442	0.409
LDL	0.012	0.003, 0.021	0.017
hs-CRP	-0.097	-0.144, -0.050	0.001

[1]Multivariate linear regression analysis. CI: Confidence interval; SBP: Systolic blood pressure; WC: Waist circumference; HbA1c: Hemoglobin A1c; LDL: Low-density lipoprotein; hs-CRP: High-sensitivity C-reactive protein.

associated with self-perceived health status in the positive group. These results may imply that a large proportion of hepatitis B patients have poor health behaviors, particularly related to smoking and alcohol consumption. Alcohol consumption and tobacco smoking are modifiable risk factors for HCC. Also, because chronic hepatitis B is an established risk factor for HCC, modification of health behaviors is a particularly important intervention for patients with chronic hepatitis B.

In males, the current smoking rate in the positive group was 41.5%, which is higher than the 23% rate in Organization for Economic Co-operation and Development (OECD) member countries[21]. In most OECD countries, the rate of daily smoking has been reduced by various measures, including stringent policies to reduce smoking and increased taxes on tobacco[21,22]. The World Health Organization estimates that smoking kills 7 million people worldwide annually, and it is the leading cause of death, illness, and impoverishment[21,23]. Nevertheless, over 60% of smokers do not quit after being diagnosed with a chronic illness for which smoking is an important prognostic factor[24]. Current smokers are at an increased risk of HCC[5]. Furthermore, there is a causal association between smoking and primary liver cancer, according to the International Agency for Research on Cancer[25]. Therefore, healthcare providers should pay more attention to the smoking habits of patients with hepatitis B. Tobacco smoking may increase the risk of HCC in several ways. First, numerous compounds in tobacco are metabolized to carcinogens in the liver. Second, tobacco smoking and chronic hepatitis B exert a synergistic effect on the risk of HCC[26]. In addition, levels of polycyclic aromatic hydrocarbons in HCC tissue are increased[27,28].

In this study, the rates of monthly and high-risk alcohol use among males were 70.4% and 17.6%, respectively, in the positive group. Heavy alcohol consumption is reportedly associated with HCC risk, although the threshold quantity/frequency is unknown[29,30]. Moreover, heavy alcohol consumption is associated with an 87% increase in the risk of HCC compared to that of non-drinkers[5]. Alcohol may contribute to hepatic

carcinogenesis *via* acetaldehyde metabolism. Acetaldehyde, the product of ethanol oxidation, may interfere with DNA synthesis and repair and increase the level of reactive oxygen species[31]. Therefore, strict abstinence should be recommended for most patients with chronic hepatitis B[29]. In addition, high-risk alcohol consumption was significantly associated with self-perceived health status in the positive group. Thus, high-risk alcohol drinkers may have a better perception of their subjective health, as reported previously[32]. Alternatively, there may be a discrepancy between the actual and perceived quantity and frequency of alcohol consumption. Further studies should investigate the relationships of these discrepancies with alcohol consumption behaviors and sex differences. Our findings imply that a meaningful proportion of patients with chronic hepatitis B consume high-risk amounts of alcohol.

HCC is being increasingly diagnosed in obese and physically inactive individuals. The development of obesity-associated HCC involves chronic inflammation induced by adipose tissue remodeling and pro-inflammatory adipokine secretion, lipotoxicity, alterations in the gut microbiota, and insulin resistance[33]. In this study, the rate of a normal body weight was high among males in the positive group. However, 32.3% of males and 26.5% of females in the positive group were obese. Physical inactivity is also associated with self-perceived health status. Obesity in patients with chronic liver disease may accelerate the development of HCC; therefore, strategies to control body weight in such patients may reduce the incidence of HCC[32].

Strengths and weaknesses of the study

The first strength of this study was its representative population, which increases the validity of our findings compared with those from hospital- or institution-based populations. Second, our findings may be helpful in the management of patients with chronic hepatitis B, because few studies have described the health habits of chronic hepatitis B patients. However, this study also had several limitations. Its cross-sectional nature prevented determination of causal relationships. In addition, our results cannot be generalized to other ethnic groups, because all of the subjects in this study were ethnic Koreans. Finally, we diagnosed hepatitis B based on the HBsAg level only, and thus repeat HBsAg testing and determination of plasma HBV DNA levels are needed to confirm the HBV infection status of the subjects.

Conclusions

In conclusion, a large percentage of Korean adults with chronic hepatitis B have poor health behaviors, particularly regarding smoking and alcohol consumption. These individuals must be encouraged to improve their health behaviors and to participate in appropriate education programs. Our findings will facilitate the development of alternative strategies to prevent HCC in patients with chronic hepatitis B.

ARTICLE HIGHLIGHTS

Research background

Chronic viral hepatitis B (CHB) is popular chronic condition in Asia-Pacific region. CHB is established risk factor of primary liver cancer that is second leading cause of cancer-related death. Health-related behaviors like as smoking, alcohol, body weight and physical activity are critical determinants of chronic illness. Most hepatitis B patients perceive that they are healthy because of asymptomatic and nonspecific nature of CHB. Such misunderstanding may cause inappropriate health behavior.

Research motivation

For the prevention of liver cancer, it seems important to manage these modifiable health behaviors. But few studies have described the health-related behaviors of chronic hepatitis B patients.

Research objectives

Our study evaluated the frequencies of five health-related behaviors (smoking, alcohol consumption, body weight, sleep duration, and physical activity) in Korean adults with CHB and association between these health behaviors and subjective health status.

Research methods

Data were obtained from 5887 subjects (2568 males, 3319 females) over 19 years old enrolled in the 2016 Korean National Health and Nutrition Examination Survey. A self-administered questionnaire and medical examination were performed to assess health-related behaviors. A chi-squared test was performed for comparisons of health behaviors between the CHB and negative groups. A *t*-test using a general linear model was used to compare self-perceived health status between the two groups.

Research results

Among males, the current smoking rate in the HBsAg positive group was higher than that in the negative group (45.5% *vs* 38.5%). In the positive group, the rates of monthly and high-risk alcohol use were 70.4% and 17.6% in males and 45.9% and 3.8% in females, respectively. The rate of alcohol use was similar between the two groups. In the HBsAg positive group, 32.3% and 49.9% of males and 26.5% and 49.6% of females were overweight and physically inactive, respectively. High-risk alcohol consumption and physical inactivity were significantly associated with self-perceived health status.

Research conclusions

Our study revealed that a large-percentage of Korean adults with chronic hepatitis B have poor health behaviors, particularly regarding tobacco smoking and alcohol consumption. These individuals must be encouraged to improve their health behaviors and to participate in appropriate education programs.

Research perspectives

These findings will facilitate the development of alternative strategies to prevent liver cancer in patients with chronic hepatitis B. Future prospective study is required to confirm our findings.

REFERENCES

1   **Schweitzer A**, Horn J, Mikolajczyk RT, Krause G, Ott JJ. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. *Lancet* 2015; **386**: 1546-1555 [PMID: 26231459 DOI: 10.1016/S0140-6736(15)61412-X]

2   **Chayanupatkul M**, Omino R, Mittal S, Kramer JR, Richardson P, Thrift AP, El-Serag HB, Kanwal F. Hepatocellular carcinoma in the absence of cirrhosis in patients with chronic hepatitis B virus infection. *J Hepatol* 2017; **66**: 355-362 [PMID: 27693539 DOI: 10.1016/j.jhep.2016.09.013]

3   **Yun EH**, Lim MK, Oh JK, Park JH, Shin A, Sung J, Park EC. Combined effect of socioeconomic status, viral hepatitis, and

lifestyles on hepatocelluar carcinoma risk in Korea. *Br J Cancer* 2010; **103**: 741-746 [PMID: 20648009 DOI: 10.1038/sj.bjc.6605803]

4   **Kimura T**, Tanaka N, Fujimori N, Sugiura A, Yamazaki T, Joshita S, Komatsu M, Umemura T, Matsumoto A, Tanaka E. Mild drinking habit is a risk factor for hepatocarcinogenesis in non-alcoholic fatty liver disease with advanced fibrosis. *World J Gastroenterol* 2018; **24**: 1440-1450 [PMID: 29632425 DOI: 10.3748/wjg.v24.i13.1440]

5   **Petrick JL**, Campbell PT, Koshiol J, Thistle JE, Andreotti G, Beane-Freeman LE, Buring JE, Chan AT, Chong DQ, Doody MM, Gapstur SM, Gaziano JM, Giovannucci E, Graubard BI, Lee IM, Liao LM, Linet MS, Palmer JR, Poynter JN, Purdue MP, Robien K, Rosenberg L, Schairer C, Sesso HD, Sinha R, Stampfer MJ, Stefanick M, Wactawski-Wende J, Zhang X, Zeleniuch-Jacquotte A, Freedman ND, McGlynn KA. Tobacco, alcohol use and risk of hepatocellular carcinoma and intrahepatic cholangiocarcinoma: The Liver Cancer Pooling Project. *Br J Cancer* 2018; **118**: 1005-1012 [PMID: 29520041 DOI: 10.1038/s41416-018-0007-z]

6   **Campbell PT**, Newton CC, Freedman ND, Koshiol J, Alavanja MC, Beane Freeman LE, Buring JE, Chan AT, Chong DQ, Datta M, Gaudet MM, Gaziano JM, Giovannucci EL, Graubard BI, Hollenbeck AR, King L, Lee IM, Linet MS, Palmer JR, Petrick JL, Poynter JN, Purdue MP, Robien K, Rosenberg L, Sahasrabuddhe VV, Schairer C, Sesso HD, Sigurdson AJ, Stevens VL, Wactawski-Wende J, Zeleniuch-Jacquotte A, Renehan AG, McGlynn KA. Body Mass Index, Waist Circumference, Diabetes, and Risk of Liver Cancer for U.S. Adults. *Cancer Res* 2016; **76**: 6076-6083 [PMID: 27742674 DOI: 10.1158/0008-5472.Can-16-0787]

7   **Andronescu CI**, Purcarea MR, Babes PA. Nonalcoholic fatty liver disease: epidemiology, pathogenesis and therapeutic implications. *J Med Life* 2018; **11**: 20-23 [PMID: 29696060]

8   **Yi SW**, Choi JS, Yi JJ, Lee YH, Han KJ. Risk factors for hepatocellular carcinoma by age, sex, and liver disorder status: A prospective cohort study in Korea. *Cancer* 2018; **124**: 2748-2757 [PMID: 29669170 DOI: 10.1002/cncr.31406]

9   **Sacerdote C**, Ricceri F. Epidemiological dimensions of the association between type 2 diabetes and cancer: A review of observational studies. *Diabetes Res Clin Pract* 2018 [PMID: 29596949 DOI: 10.1016/j.diabres.2018.03.002]

10   **Guilbert JJ**. The world health report 2002 - reducing risks, promoting healthy life. *Educ Health* (Abingdon) 2003; **16**: 230 [PMID: 14741909 DOI: 10.1080/1357628031000116808]

11   **Cramm JM**, Adams SA, Walters BH, Tsiachristas A, Bal R, Huijsman R, Rutten-Van Mölken MP, Nieboer AP. The role of disease management programs in the health behavior of chronically ill patients. *Patient Educ Couns* 2014; **95**: 137-142 [PMID: 24462120 DOI: 10.1016/j.pec.2013.12.017]

12   **Newsom JT**, Huguet N, McCarthy MJ, Ramage-Morin P, Kaplan MS, Bernier J, McFarland BH, Oderkirk J. Health behavior change following chronic illness in middle and later life. *J Gerontol B Psychol Sci Soc Sci* 2012; **67**: 279-288 [PMID: 21983040 DOI: 10.1093/geronb/gbr103]

13   **Lenfant C**. Shattuck lecture--clinical research to clinical practice--lost in translation? *N Engl J Med* 2003; **349**: 868-874 [PMID: 12944573 DOI: 10.1056/NEJMsa035507]

14   **Kaleta D**, Makowiec-Dabrowska T, Dziankowska-Zaborszczyk E, Jegier A. Physical activity and self-perceived health status. *Int J Occup Med Environ Health* 2006; **19**: 61-69 [PMID: 16881600 DOI: 10.2478/v10001-006-0005-x]

15   **Kweon S**, Kim Y, Jang MJ, Kim Y, Kim K, Choi S, Chun C, Khang YH, Oh K. Data resource profile: the Korea National Health and Nutrition Examination Survey (KNHANES). *Int J Epidemiol* 2014; **43**: 69-77 [PMID: 24585853 DOI: 10.1093/ije/dyt228]

16   **Schoenborn CA**, Vickerie JL, Barnes PM. Cigarette smoking behavior of adults: United States, 1997-98. Advance data from vital and health statistics; no 331. Hyattsville, Maryland: National Center for Health Statistics 2003; Available from: URL: https://www.cdc.gov/nchs/data/ad/ad331.pdf

17   **Bradstock K**, Forman MR, Binkin NJ, Gentry EM, Hogelin GC, Williamson DF, Trowbridge FL. Alcohol use and health behavior lifestyles among U.S. women: the behavioral risk factor surveys.

Addict Behav 1988; **13**: 61-71 [PMID: 3364225 DOI: 10.1016/0306-4603(88)90026-3]

18   **WHO Expert Consultation.** Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies. *Lancet* 2004; **363**: 157-163 [PMID: 14726171 DOI: 10.1016/s0140-6736(03)15268-3]

19   **Watson NF**, Badr MS, Belenky G, Bliwise DL, Buxton OM, Buysse D, Dinges DF, Gangwisch J, Grandner MA, Kushida C, Malhotra RK, Martin JL, Patel SR, Quan SF, Tasali E. Recommended Amount of Sleep for a Healthy Adult: A Joint Consensus Statement of the American Academy of Sleep Medicine and Sleep Research Society. *Sleep* 2015; **38**: 843-844 [PMID: 26039963 DOI: 10.5665/sleep.4716]

20   **Haskell WL**, Lee IM, Pate RR, Powell KE, Blair SN, Franklin BA, Macera CA, Heath GW, Thompson PD, Bauman A. Physical activity and public health: updated recommendation for adults from the American College of Sports Medicine and the American Heart Association. *Med Sci Sports Exerc* 2007; **39**: 1423-1434 [PMID: 17762377 DOI: 10.1249/mss.0b013e3180616b27]

21   **OECD**. Health at a Glance 2017. Available from: URL: https://www.health.gov.il/PublicationsFiles/HealthataGlance2017.pdf

22   **Bilano V**, Gilmour S, Moffiet T, d'Espaignet ET, Stevens GA, Commar A, Tuyl F, Hudson I, Shibuya K. Global trends and projections for tobacco use, 1990-2025: an analysis of smoking indicators from the WHO Comprehensive Information Systems for Tobacco Control. *Lancet* 2015; **385**: 966-976 [PMID: 25784347 DOI: 10.1016/S0140-6736(15)60264-1]

23   **GBD 2015 Risk Factors Collaborators**. Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet* 2016; **388**: 1659-1724 [PMID: 27733284 DOI: 10.1016/S0140-6736(16)31679-8]

24   **Katz DA**, Muehlenbruch DR, Brown RB, Fiore MC, Baker TB; AHRQ Smoking Cessation Guideline Study Group. Effectiveness of a clinic-based strategy for implementing the AHRQ Smoking Cessation Guideline in primary care. *Prev Med* 2002; **35**: 293-301 [PMID: 12202073 DOI: 10.1006/pmed.2002.1073]

25   **IARC Working Group on the Evaluation of Carcinogenic Risks to Humans**. Tobacco smoke and involuntary smoking. *IARC Monogr Eval Carcinog Risks Hum* 2004; **83**: 1-1438 [PMID: 15285078]

26   **Chuang SC**, Lee YC, Hashibe M, Dai M, Zheng T, Boffetta P. Interaction between cigarette smoking and hepatitis B and C virus infection on the risk of liver cancer: a meta-analysis. *Cancer Epidemiol Biomarkers Prev* 2010; **19**: 1261-1268 [PMID: 20447919 DOI: 10.1158/1055-9965.EPI-09-1297]

27   **Benowitz NL**, Hukkanen J, Jacob P 3rd. Nicotine chemistry, metabolism, kinetics and biomarkers. *Handb Exp Pharmacol* 2009; 29-60 [PMID: 19184645 DOI: 10.1007/978-3-540-69248-5_2]

28   **Chen SY**, Wang LY, Lunn RM, Tsai WY, Lee PH, Lee CS, Ahsan H, Zhang YJ, Chen CJ, Santella RM. Polycyclic aromatic hydrocarbon-DNA adducts in liver tissues of hepatocellular carcinoma patients and controls. *Int J Cancer* 2002; **99**: 14-21 [PMID: 11948486 DOI: 10.1002/ijc.10291]

29   **Iida-Ueno A**, Enomoto M, Tamori A, Kawada N. Hepatitis B virus infection and alcohol consumption. *World J Gastroenterol* 2017; **23**: 2651-2659 [PMID: 28487602 DOI: 10.3748/wjg.v23.i15.2651]

30   **Turati F**, Galeone C, Rota M, Pelucchi C, Negri E, Bagnardi V, Corrao G, Boffetta P, La Vecchia C. Alcohol and liver cancer: a systematic review and meta-analysis of prospective studies. *Ann Oncol* 2014; **25**: 1526-1535 [PMID: 24631946 DOI: 10.1093/annonc/mdu020]

31   **Seitz HK**, Stickel F. Risk factors and mechanisms of hepatocarcinogenesis with special emphasis on alcohol and oxidative stress. *Biol Chem* 2006; **387**: 349-360 [PMID: 16606331 DOI: 10.1515/bc.2006.047]

32   **Stranges S**, Notaro J, Freudenheim JL, Calogero RM, Muti P, Farinaro E, Russell M, Nochajski TH, Trevisan M. Alcohol drinking pattern and subjective health in a population-based study. *Addiction* 2006; **101**: 1265-1276 [PMID: 16911725 DOI: 10.1111/

j.1360-0443.2006.01517.x]

33   **Karagozian R**, Derdák Z, Baffy G. Obesity-associated mechanisms of hepatocarcinogenesis. *Metabolism* 2014; **63**: 607-617 [PMID: 24629562 DOI: 10.1016/j.metabol.2014.01.011]

SYSTEMATIC REVIEWS

Role of colectomy in preventing recurrent primary sclerosing cholangitis in liver transplant recipients

Bettina M Buchholz, Panagis M Lykoudis, Reena Ravikumar, Joerg M Pollok, Giuseppe K Fusai

Bettina M Buchholz, Panagis M Lykoudis, Reena Ravikumar, Joerg M Pollok, Giuseppe K Fusai, Department of HPB Surgery and Liver Transplantation, Royal Free Hospital London, London NW32QG, United Kingdom

ORCID number: Bettina M Buchholz (0000-0002-5002-3302); Panagis M Lykoudis (0000-0003-2170-0471); Reena Ravikumar (0000-0002-2302-6452); Joerg M Pollok (0000-0001-8771-4070); Giuseppe K Fusai (0000-0002-8784-6919).

Author contributions: Buchholz BM, Lykoudis PM, Ravikumar R, Pollok JM and Fusai GK designed the study; Buchholz BM and Lykoudis PM performed the literature review and data analysis; Buchholz BM, Lykoudis PM, Ravikumar R and Fusai GK interpreted the data; Buchholz BM wrote the article; Lykoudis PM, Ravikumar R, Pollok JM and Fusai GK critically revised the manuscript for intellectual content; all authors gave final approval of the final version.

Conflict-of-interest statement: The authors declare no conflict of interest.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Manuscript source: Invited manuscript

Correspondence to: Giuseppe Kito Fusai, MSc, FRCS, Surgeon, Department of HPB Surgery and Liver Transplantation, Royal Free Hospital, Pond Street, London NW32QG, United Kingdom. g.fusai@ucl.ac.uk
Telephone: +44-20-78302431
Fax: +44-20-78302688

Received: May 15, 2018

Abstract

AIM

To study the published evidence on the impact of colectomy in preventing recurrent primary sclerosing cholangitis (rPSC).

METHODS

An unrestricted systematic literature search in PubMed, EMBASE, Medline OvidSP, ISI Web of Science, Lista (EBSCO) and the Cochrane library was performed on clinical studies investigating colectomy in liver transplantation (LT) recipients with and without rPSC in the liver allograft. Study quality was evaluated according to a modification of the methodological index for non-randomized studies (MINORS) criteria. Primary endpoints were the impact of presence, timing and type of colectomy on rPSC. Overall presence of inflammatory bowel disease (IBD), time of IBD diagnosis, posttransplant IBD and immunosuppressive regimen were investigated as secondary outcome.

RESULTS

The literature search yielded a total of 180 publications. No randomized controlled trial was identified. Six retrospective studies met the inclusion criteria of which 5 studies were graded as high quality articles. Reporting of IBD was heterogenous but in four publications, either inflammatory bowel disease, ulcerative colitis or in particular active colitis post-LT significantly increased the risk of rPSC. The presence of an intact (*i.e.,* retained) colon at LT was identified as risk factor for rPSC in two of the high quality studies while four studies found no effect. Type of colectomy was not associated with rPSC but this endpoint was underreported (only in 33% of included studies).

Neither tacrolimus nor cyclosporine A yielded a significant benefit in disease recurrence of primary sclerosing cholangitis (PSC).

CONCLUSION
The data favours a protective role of pre-/peri-LT colectomy in rPSC but the current evidence is not strong enough to recommend routine colectomy for rPSC prevention.

Key words: Recurrent primary sclerosing cholangitis; Risk factor; Ulcerative colitis; Colectomy; Liver transplantation

Core tip: There are no known treatments that can alter the development and/or progression of recurrent primary sclerosing cholangitis (rPSC) after liver transplantation (LT). Shared leukocyte recruitment pathways of the gut-liver axis, bacterial translocation into the portal circulation from an inflamed gut and intestinal dysbiosis might contribute to the pathogenesis of rPSC. Indeed, inflammatory bowel disease, ulcerative colitis and in particular active colitis post-LT significantly increase the risk of rPSC and the available data favours a protective role of pre-/peri-LT colectomy in rPSC. Prospective studies and randomized trials are needed to further elucidate a possible mechanistic link between retained colon and rPSC.

INTRODUCTION

Primary sclerosing cholangitis (PSC), a chronic inflammatory disease of the liver resulting in multi-focal strictures of the biliary tree[1], is estimated to recur in 20%-25% of liver allograft recipients during the first decade after liver transplantation (LT) with a mean interval to recurrent PSC (rPSC) of 6 mo to 5 years[2,3]. The diagnosis of rPSC is usually based on the Mayo criteria consisting of histological or cholangiographic features of PSC more than 90 d after LT in the absence of hepatic artery thrombosis/ stenosis, chronic ductopenic rejection, ABO incompatibility, anastomotic biliary strictures only, or non-anastomotic biliary strictures within 90 d after LT[4].

LT remains the only therapeutic option in patients with end stage liver disease from PSC and there are no known treatments that can alter the development and/or progression of rPSC after LT which oftentimes requires re-transplantation[1,5]. Different risk factors for rPSC have been identified by individual groups: Male recipient gender, cholangiocarcinoma identified on explant histo-

logy, extended criteria donor allograft, first-degree-related donor and acute cellular rejection[6]. Although the association of ulcerative colitis (UC) and PSC is widely acknowledged[7], there is a lack of understanding with regards to the effect of UC on the risk of developing rPSC in LT recipients.

Some authors have suggested that the presence of the colon at LT is associated with an increased risk of rPSC[8]. Three recent discoveries support a mechanistic link between the colon and PSC, and the same pathways that drive PSC in the non-transplant population might lead to the development of rPSC after LT. First, intestinal inflammation may fuel hepatic inflammation *via* shared leukocyte recruitment pathways of the gut-liver axis[9]. Homing molecules such as mucosal vascular addressin cell adhesion molecule 1 (MADCAM1) and the gut-associated chemokine (C-C motif) ligand 25, which are normally restricted to the gut, are abnormally upregulated in the liver of PSC patients and facilitate the recruitment of enteric-primed destructive α4β7-positive lymphocytes into the liver[10,11]. Additionally, as a consequence of intestinal inflammation, failure of the gut mucosal barrier with translocation of enteric pathogens to the portal circulation can drive hepatic inflammation *via* epithelial pattern recognition receptor activation[9]. Third, independent from inflammatory bowel disease (IBD), intestinal dysbiosis was found to be associated with PSC, indicating that the intestinal microbiome might play a role in PSC pathogenesis[12]. This article reviews the published evidence on the role of colectomy in preventing rPSC in LT recipients.

MATERIALS AND METHODS

Literature search strategy
A comprehensive literature search was conducted using the databases of Pubmed, EMBASE, Medline OvidSP, ISI Web of Science, Lista (EBSCO) and the Cochrane library. Title, abstract, keywords and full-text of articles published in the time period from 1945 until 29th of April 2018 were screened for the search terms which were stratified in blocks with rPSC (block 1), colectomy and ulcerative colitis (block 2), and liver transplant, immunology and inflammatory bowel disease (block 3). All combinations were explored with one term each from block 1 and 2, subsequently combined with search terms from block 3 (Supplementary Figure 1). All languages and all publications on human subjects were considered. This unrestricted and unfiltered literature search was independently conducted by 2 authors (Buchholz BM and Lykoudis PM). Any disagreement was resolved by a third author (Fusai GK). The references of the identified publications were assessed for further reports pertinent to the topic.

Study selection
Articles were selected for final review if they compared LT recipients with and without rPSC, and reported at least one of the defined outcome endpoints. If the same

patient cohort was included in multiple publications, only the most recent update with the largest number of patients was retained.

Quality assessment of studies

The quality of publications identified by the above literature search was evaluated using a modification of the methodological index for non-randomized studies (MINORS) criteria, consisting of 9 items categorized into study design (clearly stated aim, inclusion of consecutive patients, prospective collection of data, endpoints appropriate to the aim of the study), data recording and data quality (appropriate follow-up period, loss to follow-up reporting, baseline equivalence of groups) and study assessment (outcome evaluation bias, adequate statistical analysis)[13]. The items were scored as 0 (not reported), 1 (reported but inadequate) or 2 (reported and adequate) with the ideal score being 18. The study evaluation for the MINORS criteria was independently performed by 2 authors (Buchholz BM and Lykoudis PM), and discrepancies were resolved by consensus with a neutral referee (Fusai GK). Given the above outlined natural history of rPSC, a follow-up time of 5 years after liver transplantation was set as appropriate.

Primary and secondary endpoints

The primary outcome was the impact of presence (yes or no), timing (pre-/peri-LT or post-LT) and type of colectomy (pan-proctocolectomy, segmental/subtotal or other) on rPSC in the liver allograft. The following endpoints were assessed as secondary outcomes: (1) Presence of IBD, time of IBD diagnosis (pre-LT, *de novo*), and post-transplant presence of IBD; and (2) primary and secondary immunosuppression. Further parameters were collected on recipient characteristics (recipient age at LT, recipient gender, MELD at LT, time to diagnosis of rPSC, and follow-up period) and donor demographics [donor age, donor gender and type of donation (DBD, DCD)]. The data of interest was extracted and tabulated from the relevant studies texts, tables and figures, by 2 independent authors (Buchholz BM and Lykoudis PM). Data are reported as n (%) and median (interquartile range) unless otherwise indicated.

Biostatistics

The statistical methods of this study were reviewed by one of the authors (Lykoudis PM) Apart of the descriptive median MINORS score, no meaningful statistical analysis was feasible in this systematic review given the heterogeneity of the statistical methods applied for the described endpoints in the included original studies.

RESULTS

The systematic literature search of the databases yielded a total of 180 publications (Figure 1). No article was identified in the Cochrane Library or Lista (EBSCO) and no additional records were retrieved from the manual search of the reference lists. Following removal of duplicates, 56 studies were screened of which 43 were excluded on title screening (*n* = 7) and abstract screening (*n* = 36) as not pertinent to the topic. The full-text of the remaining 14 publications was assessed for eligibility. Publications reporting on a smaller proportion of the same patient population as larger studies[8], conference abstracts[14,15] and cohort studies[16,17] were further excluded leaving a selection of 8 studies eligible for quality assessment.

Quality assessment of selected publications

All retrieved publications had an observational and retrospective design; no randomized controlled trial was identified. Two studies did not report any relevant endpoints and were therefore excluded in retrospect[18,19]. Hence, a total of 6 reports comparing LT recipients with and without rPSC in contemporary groups were assessed by MINORS criteria[20-25]. The median MINORS score was 11 (IQR 8.75-12.25), with incomplete outcome reporting, lack of equivalent groups and failure to adequately state loss to follow up accounting primarily for lower scores (Table 1). There was evidence in outcome evaluation bias in all but two studies[20,25]. The study design was overall good with reporting of consecutive patients in all publications and a clearly formulated study aim in most reports, but only one study reported all endpoints[25]. Follow-up period and statistical methods were appropriate apart from one study published by Gelley *et al*[22]. The latter study therefore scored overall low quality (5 points) while 5 publications were deemed of good quality with a MINORS score ranging from 10 to 13. These included single-centre experiences[20,21,24] as well as two large multi-centre cohorts[23,25]. The patient cohort of the multi-centre report by Ravikumar *et al*[25] overlaps in part with two single-centre studies[20,21] but does not completely capture the patient data; therefore, all three publications were retained.

Recipient and donor demographics

The retrieved studies on rPSC were published within the last decade and captured patient data from 1986 to 2011. The indication for LT was PSC in all patients and LT recipients were followed up for a median time of 5.7 to 9.1 years (Table 2). Follow-up was restricted by patient death but extended up to 8 to 22 years in the various studies. Diagnosis of rPSC was guided by Mayo criteria[4] in all studies and median time to diagnosis of rPSC ranged from 3.4 to 5 years as reported by 4 studies.

Demographics were analyzed and described by different statistical methods in the various studies and can therefore only be compared in a descriptive manner. rPSC was more prevalent in patients undergoing a first liver transplant at a younger age. Similarly, there was a predominance of male gender in the rPSC group, with a higher MELD at LT. Liver grafts from younger donors were also associated with rPSC. Donor gender and donor type were rarely reported; however, the available data suggested no relevant differences between groups. It is

Table 1 Methodological index for non-randomized studies criteria for selected studies on recurrent primary sclerosing cholangitis after liver transplantation

Ref.	Year	Study design				Data recording and data quality			Study assessment		MINORS score
		Study aim	Consecutive patients	Data collection	Reported endpoints	Equivalent groups	Follow-up period	Loss to follow-up	Outcome evaluation bias	Statistical methods	
Cholongitas *et al*[21]	2008	2	2	1	1	0	2	0	1	2	11
Alabraba *et al*[20]	2009	2	2	1	1	0	2	0	2	2	12
Moncrief *et al*[24]	2010	1	2	1	1	0	2	0	1	2	10
Gelley *et al*[22]	2014	1	2	1	1	0	0	0	0	0	5
Ravikumar *et al*[25]	2015	2	2	1	2	0	2	0	2	2	13
Hildebrand *et al*[23]	2016	2	2	1	1	0	2	0	1	2	11

MINORS: Methodological index for non-randomized studies.

Figure 1 PRISMA flowchart depicting literature search. References are indexed by superscript numericals.

noticeable that almost exclusively livers from donors after brain death were utilized, a fact that is likely explained by era of transplantation prior to increased transplantation of organs from donors after circulatory death.

Recipient factors such as younger age and advanced severity of liver disease were described as independent risk factors for rPSC (Table 3) but are given at the time of transplantation; it is logical that these cannot be addressed with the aim to improve outcome. The studies further suggest that a better graft selection with avoidance of donors with extended criteria, higher age and higher body mass index (BMI) could aid in preventing rPSC.

Inflammatory bowel disease

PSC develops in 2.4% to 7.5% of patients with inflammatory bowel disease (IBD) while 70% to 85% of patients with PSC will manifest symptoms of IBD during their lifetime[26]. Of the two distinct subtypes of IBD, ulcerative colitis has the strongest association with PSC accounting for 90% of the cases[7]. In line with this, data from Hildebrand *et al*[23] indicate that rates of IBD exceed the presence of UC in both rPSC and non-rPSC groups by approximately 10%. The majority of the studies (3 out of 4) stated a higher rate of IBD in LT recipients with rPSC ranging between 86% to 100% compared to the non-rPSC group (71%-79%) with the exception of Alabraba

Table 2 Demographics of study cohorts comparing liver transplantation recipients with and without recurrent primary sclerosing cholangitis in the liver graft

Ref.	Year	Study period	Number *n* (rPSC *vs* no rPSC)	Time to diagnosis of rPSC (yr)	Follow-up (yr)	Recipient age at LT (yr)	Recipient gender (male)	MELD at LT	Donor age (yr)	Donor gender (male)	Donor type (DBD)
Cholongitas *et al*[21]	2008	1989-2004	7 (13) *vs* 46 (87)	5 (0.3-10)[1]	9.1 (1-15.4)[1]	35 ± 15 *vs* 42 ± 13[2]	5 (71) *vs* 25 (54)	NR	33 ± 18 *vs* 44 ± 14[2]	6 (86) *vs* 27 (59)	NR
Alabraba *et al*[20]	2009	1986-2006	61 (23) *vs* 202 (77)	4.6 (0.5-12.9)[1]	6.9 (0-19.9)[1]	48 (16-72)[1]	50 (82) *vs* 149 (74)	NR	NR	NR	61 (100) *vs* 202 (100)
Moncrief *et al*[24]	2010	1989-2006	15 (25) *vs* 44 (75)	3.4 (1.5-5.5)	5.7 (2.8-8.8)	45 (36-53) *vs* 47 (37-52)	13 (87) *vs* 33 (75)	18 (13-21) 14 (10-21)	NR	NR	NR
Gelley *et al*[22]	2014	1995-2011	6 (24) *vs* 19 (76)	NR	NR	27 ± 7 *vs* 37 ± 12[2]	3 (50) *vs* 13 (68)	16 ± 5 *vs* 11 ± 4[2]	39 ± 14 *vs* 35 ± 11[2]	NR	NR
Ravikumar *et al*[25]	2015	1990-2010	81 (14) *vs* 484 (86)	NR	9 (5-14)	43 (34-55) *vs* 49 (41-57)	61 (75) *vs* 344 (71)	NR	39 (28-53) *vs* 43 (32-54)	46 (57) *vs* 268 (55)	80 (99) *vs* 467 (96)
Hildebrand *et al*[23]	2016	1990-2006	62 (20) *vs* 243 (80)	4.6 (0.5-14.3)[3]	8.2 (0-22)[3]	39 ± 11.5 *vs* 39 ± 10.8[2]	48 (77) *vs* 160 (66)	16 ± 6 *vs* 14 ± 7[2]	43.6 ± 16 *vs* 40.1 ± 16.9[2]	NR	NR

Data are reported as *n* (%) and median (interquartile range) unless indicated as [1]median (range), [2]mean ± SD and [3]mean (range). DBD: Donation after brain death; LT: Liver transplantation; MELD: Model of end-stage liver disease; NR: Not reported; rPSC: Recurrent primary sclerosing cholangitis.

et al[20] who observed an equivalent rate of IBD presence between groups reaching 72% and 70%, respectively (Table 4). Two studies exclusively reported on ulcerative colitis (UC) with a higher prevalence of UC in rPSC (78% -100%) *vs* non-rPSC (52%-56%)[21,25].

The available results of 4 studies on timing of IBD/ UC diagnosis demonstrates that IBD/UC occurs more frequently in LT recipients with rPSC both prior to transplantation (65%-83% *vs* 46%-74%) and *de novo* after transplantation (13%-29% *vs* 2%-6%). There is also consensus amongst reports that the overall presence of IBD/UC after LT is considerably higher in cases of rPSC (65%-100% *vs* 42%-79%). IBD, either by presence of UC post-LT[21,23,25] or severe active IBD[22,23], was reported as an independent risk factor for disease recurrence of PSC in the liver allograft in four studies which included the two multi-centre studies (Table 3).

Presence, timing and type of colectomy

None of the identified publications focused on the role of colectomy in prevention of rPSC, but some of them examined it within a broader assessment of risk factors for rPSC. Colectomy at any time was carried out at lower (7%-14% *vs* 20%-26%)[21,24] or equal rates (23%-34% *vs* 18%-32%)[20,22,25] in LT recipients with rPSC compared to the PSC cohort without recurrent disease in the liver allograft. The timing of colectomy, reported in 5 out of 6 studies, differed consistently between the two groups: colectomy in patients with rPSC was performed mainly post-LT (14%-34% *vs* 10%-13%) and less often pre- and peri-LT (0-7% *vs* 8%-21%) (Table 4). Two of the high quality studies conclude that (pre-/peri-LT) colectomy has a protective effect on rPSC in the liver allograft as the presence of a non-resected colon at transplantation was

identified as a risk factor for rPSC on univariable analysis (Table 3)[20,25]. The remaining four publications reported no significant effect of colectomy on rPSC[21-24]. Still, as a trend towards a role of colectomy in preventing rPSC was found in some of the aforementioned studies, the lack of significance may be related to a type 2 statistical error as frequently seen in underpowered studies.

Data on the type of colectomy in the identified publications is scarce and contradictory. Segmental/subtotal colectomy was more often described in the multi-centre study by Ravikumar *et al*[25] while pan-proctocolectomy (specifically post-LT) was the preferred colorectal approach in LT recipients with rPSC in the work of Alabraba *et al*[20]. Pan-proctocolectomy was compared to other forms of colectomy in which remnant colorectal tissue remained in situ (segmental/subtotal colectomies and ileoanal pouch). Disregarding timing of colectomy, the overall assessment of type of colectomy revealed no significant difference in the risk for rPSC in LT recipients in both of the aforementioned studies. However, in the subgroup of post-LT colectomies, the risk of rPSC was significantly lower in patients who had undergone either pan-proctocolectomy or subtotal colectomy compared with LT recipients with ileoanal pouch[20].

Immunosuppression

Tacrolimus and cyclosporine A were used as mainstay immunosuppression in all identified studies. In the group with rPSC, the main choice of calcineurin inhibitor (CNI) was cyclosporine A (71%-72%) in two reports[20,21] and tacrolimus (44%-67%) in three publications[22,23,25], while the two drugs were utilized in a similar fashion in the study of Moncrief *et al*[24] (Table 4). Compared to LT recipients without rPSC, cyclosporine A was more

Table 3 Summary of study outcomes on impact of colectomy on recurrent primary sclerosing cholangitis, inflammatory bowel disease-specific risk factors and non-inflammatory bowel disease-specific risk factors for recurrent primary sclerosing cholangitis

Ref.	Year	Colectomy	IBD-specific risk factor for rPSC	non-IBD risk factor for rPSC
[1]Cholongitas *et al*[21]	2008	No effect	Presence of UC post-LT	Need for maintenance steroids post-LT
Alabraba *et al*[20]	2009	Protective (pre- and peri-LT)	Presence of intact (*i.e.*, retained) colon (independent of IBD or UC)	EDC grafts
Moncrief *et al*[24]	2010	No effect	None	At least one episode of ACR CMV mismatch
Gelley *et al*[22]	2014	No effect	Severe active IBD	Higher donor BMI Younger recipient age
[1]Ravikumar *et al*[25]	2015	Protective (univariate analysis)	Presence of UC post-LT	Younger recipient age
Hildebrand *et al*[23]	2016	No effect	IBD, UC, and in particular active colitis post-LT	Higher donor age Higher INR at LT

[1]Studies reporting exclusively on ulcerative colitis. ACR: Acute cellular rejection; BMI: Body mass index; CMV: Cytomegalovirus; EDC: Extended donor criteria; IBD: Inflammatory bowel disease; INR: International normalized ratio; LT: Liver transplantation; rPSC: Recurrent primary sclerosing cholangitis; UC: Ulcerative colitis.

Table 4 Primary outcomes of study cohorts comparing liver transplantation recipients with and without recurrent primary sclerosing cholangitis in the liver graft

Ref.	Year	Presence of IBD (ever)	Time of IBD diagnosis (pre-LT/*de novo*)	Presence of IBD (post-LT)	Time of colectomy pre- and peri-LT/post-LT	Type of colectomy	Primary immunosuppression	Secondary immunosuppression
[1]Cholongitas *et al*[21]	2008	7 (100) *vs* 26 (56)	5 (71)/2 (29) *vs* 25 (54)/1 (2)	7 (100) *vs* 26 (56)	0 (0)/1 (14) *vs* 6 (13)/6 (13)	NR	TAC 2 (29) *vs* 25 (54) CyA 5 (71) *vs* 21 (46)	AZA 3 (43) *vs* 22 (48) OKT3 or ATG 2 (29) *vs* 11 (24)
Alabraba *et al*[20]	2009	39 (72) *vs* 123 (70)	NR	NR	1 (2)/14 (23) *vs* 28 (16)/18 (10)	Panproctocolectomy 7 (13) *vs* 15 (8) Segmental + subtotal 5 (9) *vs* 21 (12) Ileoanal pouch 3 (6) *vs* 10 (6)	TAC 16 (26) *vs* 104 (51) CyA 44 (72) *vs* 95 (47)	None 1 (2) *vs* 3 (2) OKT3 0 (0) *vs* 2 (2)
Moncrief *et al*[24]	2010	15 (100) *vs* 33 (75)	11 (73)/4 (27) *vs* 31 (70)/2 (5)	13 (87) *vs* 24 (55)	1 (7)/NR *vs* 9 (20)/NR	NR	TAC 7 (47) *vs* 28 (64) CyA 8 (53) *vs* 14 (32)	NR
Gelley *et al*[22]	2014	6 (100) *vs* 15 (79)	5 (83)/1 (17) *vs* 14 (74)/1 (5)	6 (100) *vs* 15 (79)	0 (0)/2 (34) *vs* 4 (21)/2 (11)	NR	TAC 4 (67) *vs* 14 (74) CyA 2 (33) *vs* 5 (26)	NR
[1]Ravikumar *et al*[25]	2015	51 (78) *vs* 220 (52)	42 (65)/9 (13) *vs* 193 (46)/27 (6)	43 (66) *vs* 181 (42)	5 (6)/14 (17) *vs* 40 (8)/46 (10)	Panproctocolectomy: 2 (2) *vs* 22 (5) Segmental + subtotal: 16 (20) *vs* 41 (8) Other: 1 (1) *vs* 17 (4)	TAC 36 (44) *vs* 330 (68) CyA 20 (25) *vs* 55 (11)	AZA 26 (32) *vs* 212 (44) MMF 10 (12) *vs* 67 (14) Steroids 38 (47) *vs* 285 (59)
Hildebrand *et al*[23]	2016	53 (86) *vs* 167 (71)	NR	48 (77) *vs* 138 (59)	NR	NR	TAC 41 (67) *vs* 150 (66) CyA 32 (53) *vs* 124 (55)	Steroids 41 (70) *vs* 133 (60)

Data are expressed as *n* (%). [1]Studies reporting exclusively on ulcerative colitis. ATG: Anti-thymocyte globulin; AZA: Azathioprine; CyA: Cyclosporine A; IBD: Inflammatory bowel disease; LT: Liver transplantation; MMF: Mycophenolate mofetil; NR: Not reported; OKT3: Muromonab-CD3; TAC: Tacrolimus.

often administered in the group with rPSC in 4 studies (25%-72% *vs* 11%-47%)[20,21,24,25] and to an equal extent in the two remaining publications (33%-53% *vs* 26%-55%)[22,23]. The choice of CNI had no effect on the risk of rPSC in 5 out of 6 studies while Ravikumar *et al*[25] found that maintenance immunosuppression with

cyclosporine A carried a 2-fold increased risk for rPSC on univariate analysis; however, the association between cyclosporine A use and rPSC was lost when adjusted for transplantation era and in the multivariable model.

Azathioprine and steroids were less frequently used long-term in LT recipients with rPSC in the multi-centre

study by Ravikumar *et al*[25], yet none of secondary immunosuppressant agents used were significantly associated with rPSC. Three further publications mention choice and frequency of secondary immunosuppression and describe no difference in the use of OKT3, anti-thymocyte globulin and steroids between the two study groups[20,21,23].

DISCUSSION

PSC is a complex liver disease characterized by chronic inflammation of the biliary epithelium[1]. The pathogenesis is not fully understood but immune dysregulation in genetically susceptible individuals is thought to play a major role[27]. Noticeably, PSC recurs in only one fifth of the transplant population[28] which implies that the natural course of PSC is altered after LT. As universally required in solid organ transplantation, management of the transplanted patient includes immunosuppression which might partially downregulate the immune pathways involved in the development of PSC. The CNI tacrolimus and cyclosporine A remain the cornerstone of modern treatment regimens to reduce allograft rejection[29]. Both drugs inhibit different stages of the T-lymphocyte and B-lymphocyte activation cycles by interfering with the interleukin-2 pathway but bind to diverse intracellular target molecules[30] and differ in potency and spectrum of immune modulation[31].

However, none of the immunosuppressive regimen utilized in the selected studies conveyed a significant benefit in disease recurrence of PSC. Assuming that the analyzed studies reflect real world practise and that no true differences exist between the two CNI affecting the development of rPSC, this would imply that both drugs either do not target the hypothesized immune mechanisms of rPSC or do so in a similar fashion. The latter seems unlikely given the fact that the use of tacrolimus is associated with increased IBD activity and the development of *de novo* IBD post-LT[32]. In line with this, a large Nordic cohort study identified tacrolimus as independent risk factor for rPSC[17]. On the other hand, bearing in mind that tacrolimus is more potent in preventing liver allograft rejection[31] and that acute cellular rejection can drive rPSC (Table 3)[24,33], one would choose tacrolimus as the preferred immunosuppressive regimen with regard to prevention of rPSC. It remains possible that the significance of cylosporine A as a risk factor for rPSC was lost in the work of Ravikumar *et al*[25] due to high statistical dependence with another variable, something that could be further investigated by Spearman's rank correlations in future studies[34].

Suggestions have been made that the selection of better quality grafts could aid in preventing PSC[22,23,25]; however, the translation of such an approach might prove difficult in the era of organ shortage as more marginal grafts are utilized to expand the donor pool[35]. It is especially recognized that the donor age has significantly increased over the past decades and continues to rise[36]. The impact of new technologies such as normothermic machine perfusion of marginal liver grafts on rPSC will

have to be awaited[37]. Yet, it is encouraging that newer studies have reported that liver grafts from donors after circulatory death can be safely utilized in LT candidates with PSC and, although the incidence of ischemic-type biliary lesions was increased in the donor group after circulatory death compared to donors after brain death, the incidence of anastomotic strictures or rPSC was not different between donor groups[38].

Considering the overall limitations in donor selection, the main targets in preventing rPSC appear to be factors in the management of the LT recipient such as frequently co-existing inflammatory bowel disease. Interestingly, it has been recently described that the coexistence of both ulcerative colitis and PSC in the same patient is associated with an increased risk of native liver disease progression and either need for liver transplantation or death[39]. Given the overall increased incidence of IBD in rPSC, it may therefore be hypothesized that the same pathways that drive PSC in the non-transplant population are engaged in the development of rPSC after LT such as shared leukocyte recruitment pathways of the gut-liver axis[10,11] and bacterial translocation into the portal circulation from an inflamed gut[9]. While it was reported that active colitis and the need for maintenance steroids (> 3 mo), mainly reflecting UC activity and not graft dysfunction, significantly predispose to rPSC[21,23], the distinct impact of disease severity of IBD on rPSC has not been well assessed in the present studies. Cholongitas *et al*[21] are the only study that reported in more detail that UC disease extension (distal or total), UC activity *per se*, and the post-LT course of UC in terms of severity, number of admissions for UC and utilization of immunosuppression for UC exacerbation was not associated with rPSC. Controversy however exists about the clinical course of pre-existing IBD post-LT in general as conflicting data either point towards ameliorated bowel disease[40,41] or reports poorly-controlled IBD exacerbation[42,43]. *De novo* IBD after LT occurs at a variable incidence of 1.3%-31.3%[42]. The 10-year risk to develop *de novo* IBD in the transplanted PSC population is estimated to be 14%-30% with a median time to onset of 4 years[44]. Even though *de novo* IBD usually presents later after transplantation and responds better to medical therapy compared to IBD recurrence[43], it has paradoxically been shown in the multi-centre study by Ravikumar *et al*[25] that the diagnosis of *de novo* UC was associated with a higher risk of rPSC compared to UC diagnosis prior LT. Liver cirrhosis attenuates T-cell function and the aforementioned finding raises the interesting question whether rPSC is driven by restored T-cell function as it has been implemented for increased IBD disease activity of previously quiescent colitis[45].

The leading indications for colectomy in patients with concomitant UC and PSC are the presence of colorectal neoplasia and severe colonic inflammation in both the pre- and post-transplant setting[32,46]. The overall necessity for colectomy due to disease activity of UC was found to be reduced in patients with advanced PSC needing liver transplantation[47]. Yet, colectomy prior to but not after PSC diagnosis is beneficial leading to a decreased

risk of liver transplantation or death[48]. It was therefore especially relevant to analyze the identified studies regarding the impact of timing and type of colectomy on rPSC as it can be hypothesized that the prognosis of the liver allograft is altered depending on exposure to the UC environment or complete eradication of intestinal inflammatory factors and the intestinal microbiome prior to graft implantation. Within the limitations of retrospective publications, the data overall supported a protective role of colectomy in rPSC if carried out prior to or at the time of LT. The fact that intestinal dysbiosis has been implemented in the pathogenesis of PSC[12] and therefore possibly also rPSC, could explain why the presence of an intact (*i.e.,* retained) colon post-LT significantly increased the risk of rPSC in the Birmingham series independently of colonic inflammation[20]. The included studies have not investigated whether the specific subpopulation of LT recipients with rPSC undergoing re-transplantation for graft failure represent a highly selected subgroup of patients that would in particular benefit from colectomy prior to or at the time of regrafting. This is something that could be investigated in future prospective studies or within the setting of a controlled study.

Peri-transplant morbidity and mortality associated to the previous colectomy procedure and complications arising from colectomy after LT were not investigated by any of the included studies. It is however known from the literature that the postoperative morbidity is high in colectomy in cirrhotic PSC patients. In particular, haemorrhagic complications and worsening liver function as well as sever liver failure requiring rescue LT are frequently encountered and are therefore of major concern[46,49,50]. Although historic reports on proctocolectomy in patients with PSC/UC have described no effect on patient survival in the pre-transplant setting[51], it is now recognized that mortality due to septic shock and hepatic failure occurs in pre-LT colectomy indicating that a simultaneous approach with colectomy at the time of transplantation might be better considered in patients with advanced PSC[46,49]. The type of colectomy appears without effect on the risk of rPSC, although one study found a higher risk of rPSC in LT recipients who underwent ileoanal pouch formation post-LT[20]. Although not reported in the selected studies and unrelated to rPSC, it should be taken into consideration that poor functional outcome as well as high rates of pouchitis (up to 90%) and pouch failure occur after ileoanal pouch formation in PSC patients[42,52].

It is a limitation of this review that only retrospective and observational reports were available to investigate the role of colectomy in rPSC. Furthermore, compared to other topics, a rather small number of studies were identified and included in our analysis. However, the selected studies were mainly of high quality and therefore reliably present the best evidence available.

In conclusion, this systematic review revealed no prospective or matched studies with comparative data on rPSC and non-rPSC. The identified retrospective, observational reports were mainly of high quality and examined the impact of colectomy on liver disease recurrence within a broader assessment of risk factors for rPSC. Within the limitations of scarce retrospective publications, the available data supports a protective role of colectomy in rPSC if carried out prior to or at the time of LT. This was indirectly underpinned by the strong association of rPSC with IBD, UC and in particular active colitis post-LT. The heterogeneity in the presentation of UC and its progression as well as the general difficulty in grading severity of IBD explain the lack of publications correlating bowel disease severity and colectomy in the development of rPSC. Choice of immunosuppression did not affect rPSC and pan-proctocolectomy was not superior compared to other forms of colectomy (ileoanal pouch and segmental-/-subtotal colectomies) in which residual colorectal tissue or intestinal microbiota could fuel immunopathogenic pathways. Taken together, the overall evidence is not strong enough to advocate routine colectomy. Prospective, matched studies and randomized trials on timing and type of colectomy in LT candidates with PSC, which should include analysis of peri-colectomy morbidity, are warranted for an adequate risk-benefit decision. However, as graft selection is limited in the era of marginal organ utilization, pre- or peri-LT timing of colectomy in LT candidates that are likely to require colectomy in due time based on IBD activity is a route that could be further explored with a view to improve outcomes after LT for PSC.

ARTICLE HIGHLIGHTS

Research background
Recurrence of PSC (rPSC) following liver transplantation occurs in up to a quarter of transplant recipients. Prophylactic colectomy has been proposed as a strategy to reduce the incidence of rPSC.

Research motivation
Current literature on the benefit of prophylactic colectomy for prevention of rPSC post liver transplantation does not include any randomized controlled trials. Findings of reported studies need thus to be examined in a critical way, to assess strength of current evidence and to highlight areas for future improvement.

Research objectives
This study aims to critically review the existing evidence regarding prophylactic colectomy for prevention of post liver transplant rPSC, to evaluate reported studies and to identify shortcomings that should be addressed in future studies.

Research methods
A systematic review was carried out, using structured search terms and a reproducible study selection procedure. Data were extracted and tabulated. The quality of the included studies was evaluated according to modified methodological index for non-randomized studies criteria.

Research results
From a total of 180 publications, 6 were included in the final analysis and all of them were retrospective cohort studies. There was significant heterogeneity in the studied samples, regarding other prognostic factors as well as timing and type of colectomy, but the overall evidence favoured a protective role of pre-/peri- liver transplantation (LT) colectomy in rPSC.

Research conclusions
This study reviews and reports the results of the existing literature in a systematic and objective way. In the absence of randomized prospective studies, such an approach is indicated for drawing conclusions based on findings of retrospective cohort studies. It confirms the overall impression that colectomy might convey protection against rPSC after LT, but the current literature cannot provide definite

answers. Finally, our work identifies a lack of comparable groups and failure to report loss to follow-up as the main limitations of reported studies.

Research perspectives

According to the findings of the present study, prophylactic colectomy seems to play a protective role in rPSC post LT, but the existing evidence is not strong. The question would be better answered through prospective randomized trials. It is understood though that such attempt might face several difficulties, particularly in terms of sample size. Alternatively, if retrospective studies were to be carried out, they should include comparison between two groups, those who undergo prophylactic colectomy and those who don't, and patients' characteristics, follow-up and outcomes should be reported in a more detailed way.

REFERENCES

1   **Eaton JE**, Talwalkar JA, Lazaridis KN, Gores GJ, Lindor KD. Pathogenesis of primary sclerosing cholangitis and advances in diagnosis and management. *Gastroenterology* 2013; **145**: 521-536 [PMID: 23827861 DOI: 10.1053/j.gastro.2013.06.052]

2   **Chapman R**, Fevery J, Kalloo A, Nagorney DM, Boberg KM, Shneider B, Gores GJ; American Association for the Study of Liver Diseases. Diagnosis and management of primary sclerosing cholangitis. *Hepatology* 2010; **51**: 660-678 [PMID: 20101749 DOI: 10.1002/hep.23294]

3   **Duclos-Vallee JC**, Sebagh M. Recurrence of autoimmune disease, primary sclerosing cholangitis, primary biliary cirrhosis, and autoimmune hepatitis after liver transplantation. *Liver Transpl* 2009; **15** Suppl 2: S25-S34 [PMID: 19876939 DOI: 10.1002/lt.21916]

4   **Graziadei IW**, Wiesner RH, Batts KP, Marotta PJ, LaRusso NF, Porayko MK, Hay JE, Gores GJ, Charlton MR, Ludwig J, Poterucha JJ, Steers JL, Krom RA. Recurrence of primary sclerosing cholangitis following liver transplantation. *Hepatology* 1999; **29**: 1050-1056 [PMID: 10094945 DOI: 10.1002/hep.510290427]

5   **Karlsen TH**, Folseraas T, Thorburn D, Vesterhus M. Primary sclerosing cholangitis - a comprehensive review. *J Hepatol* 2017; **67**: 1298-1323 [PMID: 28802875 DOI: 10.1016/j.jhep.2017.07.022]

6   **Carbone M**, Neuberger JM. Autoimmune liver disease, autoimmunity and liver transplantation. *J Hepatol* 2014; **60**: 210-223 [PMID: 24084655 DOI: 10.1016/j.jhep.2013.09.020]

7   **Loftus EV Jr**, Harewood GC, Loftus CG, Tremaine WJ, Harmsen WS, Zinsmeister AR, Jewell DA, Sandborn WJ. PSC-IBD: a unique form of inflammatory bowel disease associated with primary sclerosing cholangitis. *Gut* 2005; **54**: 91-96 [PMID: 15591511 DOI: 10.1136/gut.2004.046615]

8   **Vera A**, Moledina S, Gunson B, Hubscher S, Mirza D, Olliff S, Neuberger J. Risk factors for recurrence of primary sclerosing cholangitis of liver allograft. *Lancet* 2002; **360**: 1943-1944 [PMID: 12493264 DOI: 10.1016/S0140-6736(02)11861-7]

9   **Trivedi PJ**, Adams DH. Gut-liver immunity. *J Hepatol* 2016; **64**: 1187-1189 [PMID: 26686270 DOI: 10.1016/j.jhep.2015.12.002]

10 **Grant AJ**, Lalor PF, Hübscher SG, Briskin M, Adams DH. MAdCAM-1 expressed in chronic inflammatory liver disease supports mucosal lymphocyte adhesion to hepatic endothelium (MAdCAM-1 in chronic inflammatory liver disease). *Hepatology* 2001; **33**: 1065-1072 [PMID: 11343233 DOI: 10.1053/jhep.2001.24231]

11 **Eksteen B**, Grant AJ, Miles A, Curbishley SM, Lalor PF, Hübscher SG, Briskin M, Salmon M, Adams DH. Hepatic endothelial CCL25 mediates the recruitment of CCR9+ gut-homing lymphocytes to the liver in primary sclerosing cholangitis. *J Exp Med* 2004; **200**: 1511-1517 [PMID: 15557349 DOI: 10.1084/jem.20041035]

12 **Sabino J**, Vieira-Silva S, Machiels K, Joossens M, Falony G, Ballet V, Ferrante M, Van Assche G, Van der Merwe S, Vermeire S, Raes J. Primary sclerosing cholangitis is characterised by intestinal dysbiosis independent from IBD. *Gut* 2016; **65**: 1681-1689 [PMID: 27207975 DOI: 10.1136/gutjnl-2015-311004]

13 **Slim K**, Nini E, Forestier D, Kwiatkowski F, Panis Y, Chipponi J. Methodological index for non-randomized studies (minors): development and validation of a new instrument. *ANZ J Surg* 2003; **73**: 712-716 [PMID: 12956787 DOI: 10.1046/j.1445-2197.2003.02748.x]

14 **Srivasta B**, Rushbrook S, Gelson W, Alexander G. Outcomes of liver transplantation in primary sclerosing cholangitis (PSC) - a single UK centre experience. *J Hepatol* 2011; **54**: S363-S534. [DOI: 10.1016/S0168-8278(11)61316-0]

15 **Dekkers N**, Meeteren MWV, Inderson A, Laleman W, Desschans B, Hoek BV, Jong MD, Sabino J, Sarasqueta AF, Vermeire S. Does mucosal inflammation drive recurrence of PSC in liver transplant recipients with ulcerative colitis? J Crohn's Colitis 2018; **12**: P216. [DOI: 10.1093/ecco-jcc/jjx180.343]

16 **Graziadei IW**, Wiesner RH, Marotta PJ, Porayko MK, Hay JE, Charlton MR, Poterucha JJ, Rosen CB, Gores GJ, LaRusso NF, Krom RA. Long-term results of patients undergoing liver transplantation for primary sclerosing cholangitis. *Hepatology* 1999; **30**: 1121-1127 [PMID: 10534330 DOI: 10.1002/hep.510300501]

17 **Lindström L**, Jørgensen KK, Boberg KM, Castedal M, Rasmussen A, Rostved AA, Isoniemi H, Bottai M, Bergquist A. Risk factors and prognosis for recurrent primary sclerosing cholangitis after liver transplantation: a Nordic Multicentre Study. *Scand J Gastroenterol* 2018; **53**: 297-304 [PMID: 29301479 DOI: 10.1080/00365521.2017.1421705]

18 **Gordon FD**, Goldberg DS, Goodrich NP, Lok AS, Verna EC, Selzner N, Stravitz RT, Merion RM. Recurrent primary sclerosing cholangitis in the Adult-to-Adult Living Donor Liver Transplantation Cohort Study: Comparison of risk factors between living and deceased donor recipients. *Liver Transpl* 2016; **22**: 1214-1222 [PMID: 27339253 DOI: 10.1002/lt.24496]

19 **Joshi D**, Bjarnason I, Belgaumkar A, O'Grady J, Suddle A, Heneghan MA, Aluvihare V, Rela M, Heaton N, Agarwal K. The impact of inflammatory bowel disease post-liver transplantation for primary sclerosing cholangitis. *Liver Int* 2013; **33**: 53-61 [PMID: 22103794 DOI: 10.1111/j.1478-3231.2011.02677.x]

20 **Alabraba E**, Nightingale P, Gunson B, Hubscher S, Olliff S, Mirza D, Neuberger J. A re-evaluation of the risk factors for the recurrence of primary sclerosing cholangitis in liver allografts. *Liver Transpl* 2009; **15**: 330-340 [PMID: 19243003 DOI: 10.1002/lt.21679]

21 **Cholongitas E**, Shusang V, Papatheodoridis GV, Marelli L, Manousou P, Rolando N, Patch D, Rolles K, Davidson B, Burroughs AK. Risk factors for recurrence of primary sclerosing cholangitis after liver transplantation. *Liver Transpl* 2008; **14**: 138-143 [PMID: 18236447 DOI: 10.1002/lt.21260]

22 **Gelley F**, Zádori G, Görög D, Kóbori L, Fehérvári I, Gámán G, Gerlei Z, Nagy P, Sárváry E, Nemes B. Recurrence of primary sclerosing cholangitis after liver transplantation - The Hungarian experience. *Interv Med Appl Sci* 2014; **6**: 16-18 [PMID: 24672670 DOI: 10.1556/IMAS.6.2014.1.2]

23 **Hildebrand T**, Pannicke N, Dechene A, Gotthardt DN, Kirchner G, Reiter FP, Sterneck M, Herzer K, Lenzen H, Rupp C, Barg-Hock H, de Leuw P, Teufel A, Zimmer V, Lammert F, Sarrazin C, Spengler U, Rust C, Manns MP, Strassburg CP, Schramm C, Weismüller TJ; German PSC Study Group. Biliary strictures and recurrence after liver transplantation for primary sclerosing cholangitis: A retrospective multicenter analysis. *Liver Transpl* 2016; **22**: 42-52 [PMID: 26438008 DOI: 10.1002/lt.24350]

24 **Moncrief KJ**, Savu A, Ma MM, Bain VG, Wong WW, Tandon P. The natural history of inflammatory bowel disease and primary sclerosing cholangitis after liver transplantation--a single-centre experience. *Can J Gastroenterol* 2010; **24**: 40-46 [PMID: 20186355 DOI: 10.1155/2010/830291]

25 **Ravikumar R**, Tsochatzis E, Jose S, Allison M, Athale A, Creamer F, Gunson B, Iyer V, Madanur M, Manas D, Monaco A, Mirza D, Owen N, Roberts K, Sen G, Srinivasan P, Wigmore S, Fusai G, Fernando B, Burroughs A. Risk factors for recurrent primary sclerosing cholangitis after liver transplantation. *J Hepatol* 2015; **63**: 1139-1146 [PMID: 26186988 DOI: 10.1016/j.jhep.2015.07.005]

26 **Broomé U**, Glaumann H, Lindstöm E, Lööf L, Almer S, Prytz H, Sandberg-Gertzén H, Lindgren S, Fork FT, Järnerot G, Olsson R. Natural history and outcome in 32 Swedish patients with small duct primary sclerosing cholangitis (PSC). *J Hepatol* 2002; **36**: 586-589

[PMID: 11983440 DOI: 10.1016/S0168-8278(02)00036-3]

27   **Gidwaney NG**, Pawa S, Das KM. Pathogenesis and clinical spectrum of primary sclerosing cholangitis. *World J Gastroenterol* 2017; **23**: 2459-2469 [PMID: 28465630 DOI: 10.3748/wjg.v23.i14.2459]

28   **Carbone M**, Neuberger J. Liver transplantation in PBC and PSC: indications and disease recurrence. *Clin Res Hepatol Gastroenterol* 2011; **35**: 446-454 [PMID: 21459072 DOI: 10.1016/j.clinre.2011.02.007]

29   **Rush D**. The impact of calcineurin inhibitors on graft survival. *Transplant Rev* (Orlando) 2013; **27**: 93-95 [PMID: 23743217 DOI: 10.1016/j.trre.2013.04.003]

30   **Stepkowski SM**. Molecular targets for existing and novel immunosuppressive drugs. *Expert Rev Mol Med* 2000; **2**: 1-23 [PMID: 14585137 DOI: 10.1017/S1462399400001769]

31   **McAlister VC**, Haddad E, Renouf E, Malthaner RA, Kjaer MS, Gluud LL. Cyclosporin versus tacrolimus as primary immunosuppressant after liver transplantation: a meta-analysis. *Am J Transplant* 2006; **6**: 1578-1585 [PMID: 16827858 DOI: 10.1111/j.1600-6143.2006.01360.x]

32   **Jørgensen KK**, Lindström L, Cvancarova M, Karlsen TH, Castedal M, Friman S, Schrumpf E, Foss A, Isoniemi H, Nordin A, Holte K, Rasmussen A, Bergquist A, Vatn MH, Boberg KM. Immunosuppression after liver transplantation for primary sclerosing cholangitis influences activity of inflammatory bowel disease. *Clin Gastroenterol Hepatol* 2013; **11**: 517-523 [PMID: 23333218 DOI: 10.1016/j.cgh.2012.12.027]

33   **Kugelmas M**, Spiegelman P, Osgood MJ, Young DA, Trotter JF, Steinberg T, Wachs ME, Bak T, Kam I, Everson GT. Different immunosuppressive regimens and recurrence of primary sclerosing cholangitis after liver transplantation. *Liver Transpl* 2003; **9**: 727-732 [PMID: 12827560 DOI: 10.1053/jlts.2003.50143]

34   **Lehman A,** O'Rourke N, Hatcher L, Stepanski E. JMP for basic univariate and multivariate statistics: a step-by-step guide. Cary, NC: SAS, 2005.

35   **Attia M**, Silva MA, Mirza DF. The marginal liver donor--an update. *Transpl Int* 2008; **21**: 713-724 [PMID: 18492121 DOI: 10.1111/j.1432-2277.2008.00696.x]

36   **Jochmans I**, van Rosmalen M, Pirenne J, Samuel U. Adult Liver Allocation in Eurotransplant. *Transplantation* 2017; **101**: 1542-1550 [PMID: 28060242 DOI: 10.1097/TP.0000000000001631]

37   **Ravikumar R**, Leuvenink H, Friend PJ. Normothermic liver preservation: a new paradigm? *Transpl Int* 2015; **28**: 690-699 [PMID: 25847684 DOI: 10.1111/tri.12576]

38   **Trivedi PJ**, Scalera I, Slaney E, Laing RW, Gunson B, Hirschfield GM, Schlegel A, Ferguson J, Muiesan P. Clinical outcomes of donation after circulatory death liver transplantation in primary sclerosing cholangitis. *J Hepatol* 2017; **67**: 957-965 [PMID: 28690174 DOI: 10.1016/j.jhep.2017.06.027]

39   **Weismüller TJ**, Trivedi PJ, Bergquist A, Imam M, Lenzen H, Ponsioen CY, Holm K, Gotthardt D, Färkkilä MA, Marschall HU, Thorburn D, Weersma RK, Fevery J, Mueller T, Chazouillères O, Schulze K, Lazaridis KN, Almer S, Pereira SP, Levy C, Mason A, Naess S, Bowlus CL, Floreani A, Halilbasic E, Yimam KK, Milkiewicz P, Beuers U, Huynh DK, Pares A, Manser CN, Dalekos GN, Eksteen B, Invernizzi P, Berg CP, Kirchner GI, Sarrazin C, Zimmer V, Fabris L, Braun F, Marzioni M, Juran BD, Said K, Rupp C, Jokelainen K, Benito de Valle M, Saffioti F, Cheung A, Trauner M, Schramm C, Chapman RW, Karlsen TH, Schrumpf E, Strassburg CP, Manns MP, Lindor KD, Hirschfield GM, Hansen BE, Boberg KM; International PSC Study Group. Patient Age, Sex, and Inflammatory Bowel Disease Phenotype Associate With Course of Primary Sclerosing Cholangitis. *Gastroenterology* 2017; **152**: 1975-1984.e8 [PMID: 28274849 DOI: 10.1053/j.gastro.2017.02.038]

40   **Navaneethan U**, Choudhary M, Venkatesh PG, Lashner BA, Remzi FH, Shen B, Kiran RP. The effects of liver transplantation on the clinical course of colitis in ulcerative colitis patients with primary sclerosing cholangitis. *Aliment Pharmacol Ther* 2012; **35**: 1054-1063 [PMID: 22428731 DOI: 10.1111/j.1365-2036.2012.05067.x]

41   **Fattahi MR**, Malek-Hosseini SA, Sivandzadeh GR, Safarpour AR, Bagheri Lankarani K, Taghavi AR, Ejtehadi F. Clinical Course of Ulcerative Colitis After Liver Transplantation in Patients with Concomitant Primary Sclerosing Cholangitis and Ulcerative Colitis. *Inflamm Bowel Dis* 2017; **23**: 1160-1167 [PMID: 28520586 DOI: 10.1097/MIB.0000000000001105]

42   **de Vries AB**, Janse M, Blokzijl H, Weersma RK. Distinctive inflammatory bowel disease phenotype in primary sclerosing cholangitis. *World J Gastroenterol* 2015; **21**: 1956-1971 [PMID: 25684965 DOI: 10.3748/wjg.v21.i6.1956]

43   **Verdonk RC**, Dijkstra G, Haagsma EB, Shostrom VK, Van den Berg AP, Kleibeuker JH, Langnas AN, Sudan DL. Inflammatory bowel disease after liver transplantation: risk factors for recurrence and *de novo* disease. *Am J Transplant* 2006; **6**: 1422-1429 [PMID: 16686766 DOI: 10.1111/j.1600-6143.2006.01333.x]

44   **Indriolo A**, Ravelli P. Clinical management of inflammatory bowel disease in the organ recipient. *World J Gastroenterol* 2014; **20**: 3525-3533 [PMID: 24707135 DOI: 10.3748/wjg.v20.i13.3525]

45   **Rust C**, Brand S. PSC: Protect and serve with colitis: does it help the liver to have severe ulcerative colitis? *Gut* 2011; **60**: 1165-1166 [PMID: 21561875 DOI: 10.1136/gut.2011.240309]

46   **Treeprasertsuk S**, Björnsson E, Sinakos E, Weeding E, Lindor KD. Outcome of patients with primary sclerosing cholangitis and ulcerative colitis undergoing colectomy. *World J Gastrointest Pharmacol Ther* 2013; **4**: 61-68 [PMID: 23919218 DOI: 10.4292/wjgpt.v4.i3.61]

47   **Navaneethan U**, Venkatesh PG, Mukewar S, Lashner BA, Remzi FH, McCullough AJ, Kiran RP, Shen B, Fung JJ. Progressive primary sclerosing cholangitis requiring liver transplantation is associated with reduced need for colectomy in patients with ulcerative colitis. *Clin Gastroenterol Hepatol* 2012; **10**: 540-546 [PMID: 22245961 DOI: 10.1016/j.cgh.2012.01.006]

48   **Nordenvall C**, Olén O, Nilsson PJ, von Seth E, Ekbom A, Bottai M, Myrelid P, Bergquist A. Colectomy prior to diagnosis of primary sclerosing cholangitis is associated with improved prognosis in a nationwide cohort study of 2594 PSC-IBD patients. *Aliment Pharmacol Ther* 2018; **47**: 238-245 [PMID: 29064110 DOI: 10.1111/apt.14393]

49   **Lian L**, Menon KV, Shen B, Remzi F, Kiran RP. Inflammatory bowel disease complicated by primary sclerosing cholangitis and cirrhosis: is restorative proctocolectomy safe? *Dis Colon Rectum* 2012; **55**: 79-84 [PMID: 22156871 DOI: 10.1097/DCR.0b013e3182315745]

50   **Poritz LS**, Koltun WA. Surgical management of ulcerative colitis in the presence of primary sclerosing cholangitis. *Dis Colon Rectum* 2003; **46**: 173-178 [PMID: 12576890 DOI: 10.1007/s10350-004-6520-6]

51   **Cangemi JR**, Wiesner RH, Beaver SJ, Ludwig J, MacCarty RL, Dozois RR, Zinsmeister AR, LaRusso NF. Effect of proctocolectomy for chronic ulcerative colitis on the natural history of primary sclerosing cholangitis. *Gastroenterology* 1989; **96**: 790-794 [PMID: 2914641 DOI: 10.1016/S0016-5085(89)80078-2]

52   **Block M**, Jørgensen KK, Oresland T, Lindholm E, Grzyb K, Cvancarova M, Vatn MH, Boberg KM, Börjesson L. Colectomy for patients with ulcerative colitis and primary sclerosing cholangitis - what next? *J Crohns Colitis* 2014; **8**: 421-430 [PMID: 24239402 DOI: 10.1016/j.crohns.2013.10.008]

META-ANALYSIS

Hepatitis B reactivation in patients receiving direct-acting antiviral therapy or interferon-based therapy for hepatitis C: A systematic review and meta-analysis

Xian-Wan Jiang, Jian-Zhong Ye, Ya-Ting Li, Lan-Juan Li

Xian-Wan Jiang, Jian-Zhong Ye, Ya-Ting Li, Lan-Juan Li, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

Xian-Wan Jiang, Jian-Zhong Ye, Ya-Ting Li, Lan-Juan Li, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Hangzhou 310003, Zhejiang Province, China

ORCID number: Xian-Wan Jiang (0000-0003-0982-7306); Jian-Zhong Ye (0000-0001-8174-2580); Ya-Ting Li (0000-0002-0761-4967); Lan-Juan Li (0000-0001-6945-0593).

Correspondence to: Lan-Juan Li, MD, PhD, Professor, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, School of Medicine, Zhejiang University, No. 79 Qingchun Road, Hangzhou 310003, Zhejiang Province, China. ljli@zju.edu.cn
Telephone: +86-571-87236759
Fax: +86-571-87236459

Abstract

AIM
To assess the incidence of hepatitis B virus (HBV) reactivation in patients receiving direct-acting antiviral agent (DAA)-based therapy or interferon (IFN)-based therapy for hepatitis C and the effectiveness of preemptive anti-HBV therapy for preventing HBV reactivation.

METHODS
The PubMed, MEDLINE and EMBASE databases were searched, and 39 studies that reported HBV reactivation in HBV/hepatitis C virus coinfected patients receiving DAA-based therapy or IFN-based therapy were included. The primary outcome was the rate of HBV reactivation. The secondary outcomes included HBV reactivation-related hepatitis and the effectiveness of preemptive anti-HBV treatment with nucleos(t)ide analogues. The pooled effects were assessed using a random effects model.

RESULTS
The rate of HBV reactivation was 21.1% in hepatitis B

surface antigen (HBsAg)-positive patients receiving DAA-based therapy and 11.9% in those receiving IFN-based therapy. The incidence of hepatitis was lower in HBsAg-positive patients with undetectable HBV DNA compared to patients with detectable HBV DNA receiving DAA therapy (RR = 0.20, 95%CI: 0.06-0.64, *P* = 0.007). The pooled HBV reactivation rate in patients with previous HBV infection was 0.6% for those receiving DAA-based therapy and 0 for those receiving IFN-based therapy, and none of the patients experienced a hepatitis flare related to HBV reactivation. Preemptive anti-HBV treatment significantly reduced the potential risk of HBV reactivation in HBsAg-positive patients undergoing DAA-based therapy (RR = 0.31, 95%CI: 0.1-0.96, *P* = 0.042).

CONCLUSION
The rate of HBV reactivation and hepatitis flare occurrence is higher in HBsAg-positive patients receiving DAA-based therapy than in those receiving IFN-based therapy, but these events occur less frequently in patients with previous HBV infection. Preemptive anti-HBV treatment is effective in preventing HBV reactivation.

Key words: Hepatitis C; Hepatitis B virus reactivation; Coinfection; Direct-acting antiviral agents; Meta-analysis

Core tip: We assessed the potential risk of hepatitis B virus (HBV) reactivation in patients receiving direct-acting antiviral agent (DAA)-based therapy or interferon-based therapy for hepatitis C. Preemptive anti-HBV treatment proved to be effective in preventing HBV reactivation during DAA therapy. These findings support the use of entecavir or tenofovir in hepatitis B surface antigen-positive patients prior to the initiation of DAA therapy.

INTRODUCTION

Hepatitis B virus/hepatitis C virus (HBV/HCV) dual infection is common in regions with a high HBV prevalence, as HBV and HCV share a similar mode of transmission[1]. The global prevalence of HBV/HCV dual infection is approximately 5%-10% in patients with chronic HCV infection, and the prevalence is reported to be 8.4% in China and 12%-14% in East Asia[2]. HBV/HCV-coinfected patients tend to have more severe liver fibrosis and a higher risk of hepatocellular carcinoma than those without coinfection[3].

Recently, interferon (IFN)-based treatment for HCV infection has been gradually replaced by direct-acting antiviral agent (DAA)-based therapy due to the higher HCV sustained virologic response (SVR) rate and greater tolerability of DAA-based therapy. However, concerns have been raised regarding HBV reactivation in patients receiving DAA therapy. HBV reactivation may occur after immunosuppression therapy and chemotherapy and may result in reactivation-related hepatitis, liver failure, need for liver transplantation, and even death[4]. Similarly, HBV reactivation can occur in HBV/HCV-coinfected patients undergoing IFN-based or DAA-based treatment[5]. The United States Food and Drug Administration (FDA) has reported 29 cases of HBV reactivation, and liver failure occurred in three of these cases after DAA-based treatment[6]. As a result, the FDA introduced a black box warning regarding HBV reactivation risk in HBV/HCV-coinfected patients receiving DAA therapy[7].

A previous meta-analysis reported the proportion of HBV reactivation in HBV/HCV-coinfected individuals, but only two of the included publications involved HBV reactivation with DAA-based therapy. Moreover, the need for preemptive anti-HBV therapy remains controversial[8,9]. Therefore, we conducted a comprehensive meta-analysis to assess the incidence of hepatitis B reactivation in patients receiving DAA-based therapy or IFN-based therapy and the effectiveness of preemptive anti-HBV therapy for preventing HBV reactivation.

MATERIALS AND METHODS

Data search strategy
This systematic review was based on published literature and included 39 studies. Figure 1 shows the literature search process for the meta-analysis. Relevant publications were extracted from the PubMed, MEDLINE and EMBASE databases from inception to December 30, 2017 using the following key words and subject terms: hepatitis B virus, hepatitis C virus, HBV and HCV dual infection, coinfection and reactivation. The data search was not limited by language or study type. In addition, the reference lists of relevant reviews and online conference abstracts were also screened. When two publications investigated the same population, only the most recent study or the more detailed study was included. Our review adhered to the PRISMA guidelines[10], and the protocol was registered on PROSPERO (registration number: CRD42018085920).

Study selection
Studies included in the meta-analysis were required to meet the following criteria: (1) Conducted in patients treated with DAA-based therapy or IFN-based therapy; and (2) involved with HBV reactivation or HCV SVR after anti-HCV treatment. The following studies were excluded: (1) Studies in patients with human immunodeficiency virus coinfection; and (2) case reports and studies with no extractable data.
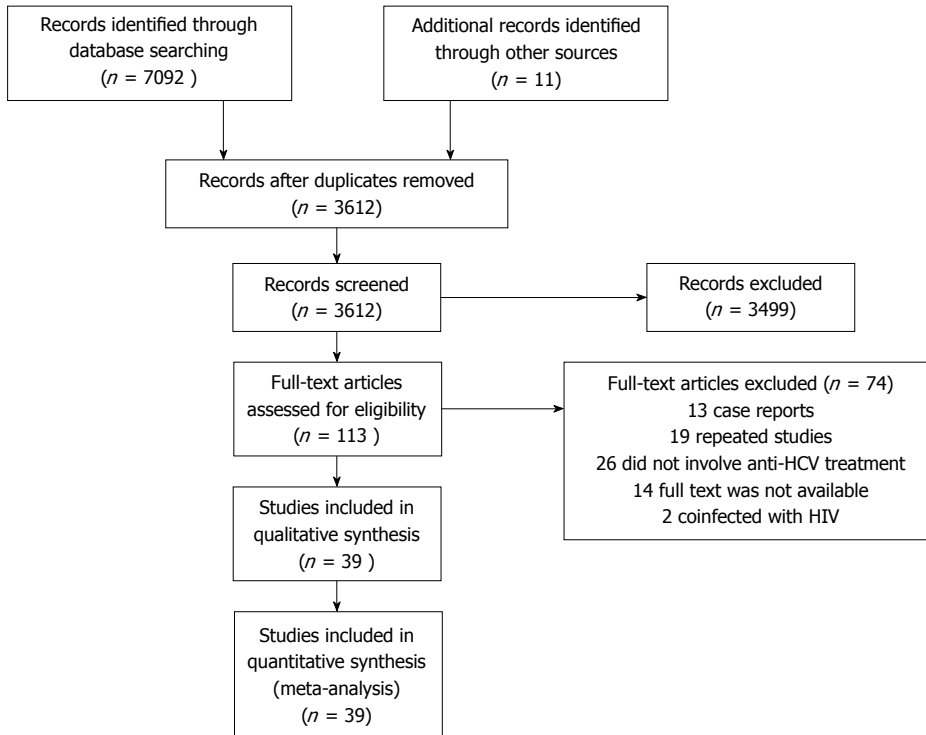
Figure 1 Literature search process for the systematic review and meta-analysis. HCV: Hepatitis C virus; HIV: Human immunodeficiency virus.

The primary outcome was HBV reactivation in accordance with the American Association for the Study of Liver Diseases (AASLD) criteria[11]: (1) Chronic HBV infection (HBsAg-positive; increase in HBV DNA > 2 log IU/mL from baseline level or HBV DNA > 100 IU/mL in patients with previously undetectable HBV DNA); and (2) previous HBV infection (HBsAg-negative, HBcAb-positive; HBsAg changed from negative to positive or HBV DNA ⩾ 20 IU/mL during treatment). The secondary outcomes included HBV reactivation-related hepatitis, the HCV SVR at the end of anti-HCV treatment and the efficacy of pre-emptive anti-HBV treatment with nucleos(t)ide analogues (NUCs). A hepatitis flare was defined as a concomitant increase in alanine aminotransferase of at least twice the upper limit of normal.

Data extraction and quality assessment

Qualitative studies were selected for the meta-analysis, and both reviews and case reports were excluded. All data were extracted by two independent investigators. The data included the following: Study design, study year, country, patient age and sex, total number of patients treated, number of patients with HBV reactivation and reactivation-related hepatitis, number of patients with SVR, HBV DNA level and HBV marker status at baseline, and treatment regimen and duration.

The quality assessment tools (Systematic Evidence Review from the Risk Assessment Work Group) from the National Institutes of Health were used to assess the methodological quality of the cohort studies and randomized controlled trials. Each study was assessed as "good", "fair", or "poor" quality using the items included

in each tool. Studies of "poor" quality suggested a high risk of bias[12].

Statistical analysis

The meta-analysis was conducted in Stata version 14.0 (StataCorp, College Station, TX, United States) using the metan, metaprop, metareg and metabias commands. The incidence rates of HBV reactivation and hepatitis flare were calculated using the random effects model, and the Freeman-Tukey double arcsine transformation was used to stabilize the variance of the raw data[13]. The pooled relative risk (RR) with 95%CIs for HBV reactivation and hepatitis flare was calculated according to the HBV DNA level with an inverse variance approach. The heterogeneity in the pooled studies was estimated with the I^2 statistic. Significant heterogeneity was investigated using meta-regression and subgroup analyses for treatment regimen (IFN-based therapy *vs* DAA-based therapy), HBV DNA level (detectable HBV DNA *vs* undetectable HBV DNA), study sample size ($n < 30$ *vs* $n \geqslant 30$) and race (Asian *vs* non-Asian). A two-sided *P* value less than 0.05 was regarded as statistically significant. Publication bias was tested using Egger's test and was assessed by funnel plots.

RESULTS

Study characteristics and quality assessment

The systematic review identified 7092 articles. After the elimination of duplicates, the titles and abstracts were screened, and 39 full articles met the inclusion criteria. These studies were conducted between 1997 and 2017,
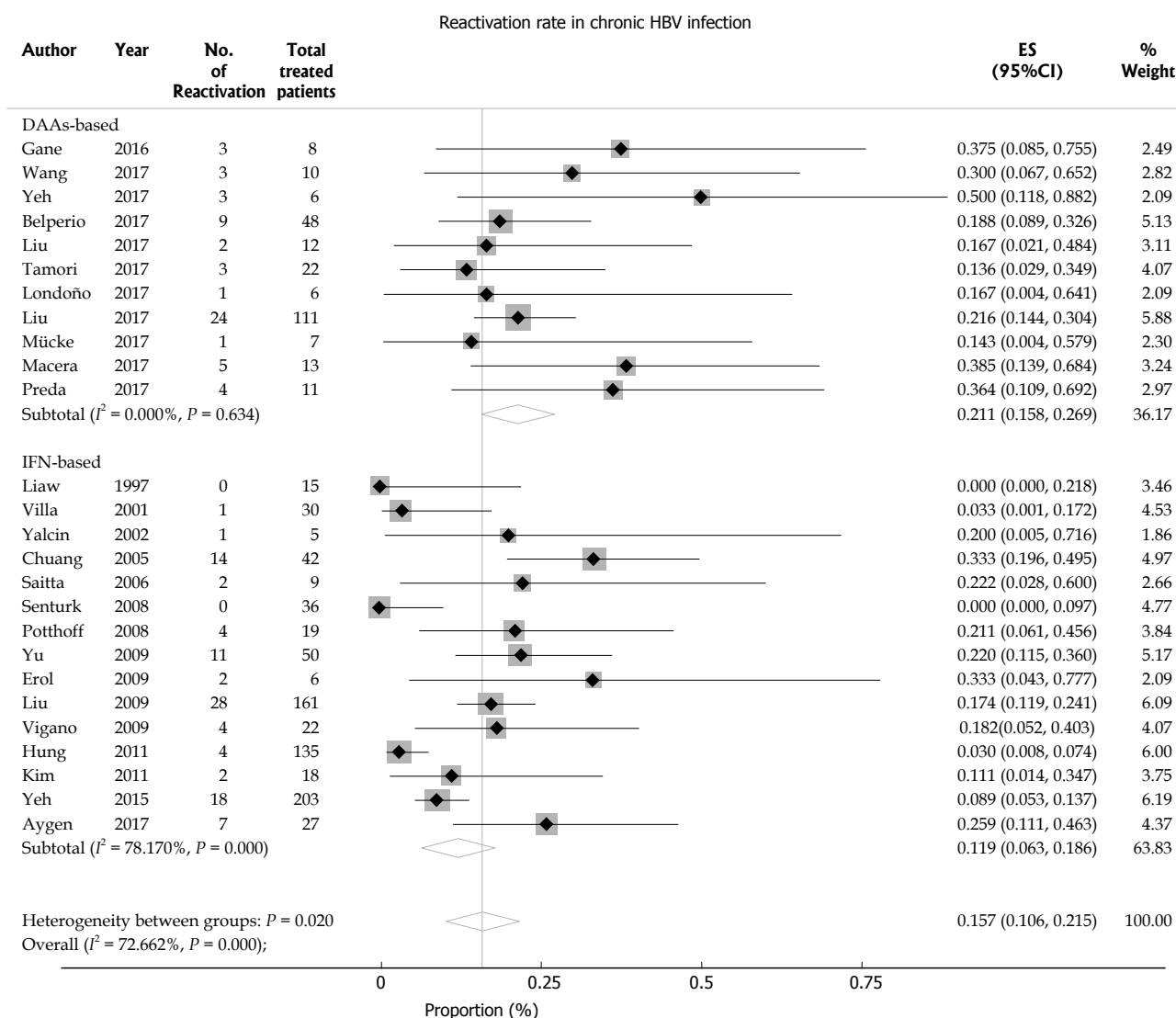
Reactivation rate in chronic HBV infection

Author	Year	No. of Reactivation	Total treated patients		ES (95%CI)	% Weight
DAAs-based						
Gane	2016	3	8		0.375 (0.085, 0.755)	2.49
Wang	2017	3	10		0.300 (0.067, 0.652)	2.82
Yeh	2017	3	6		0.500 (0.118, 0.882)	2.09
Belperio	2017	9	48		0.188 (0.089, 0.326)	5.13
Liu	2017	2	12		0.167 (0.021, 0.484)	3.11
Tamori	2017	3	22		0.136 (0.029, 0.349)	4.07
Londoño	2017	1	6		0.167 (0.004, 0.641)	2.09
Liu	2017	24	111		0.216 (0.144, 0.304)	5.88
Mücke	2017	1	7		0.143 (0.004, 0.579)	2.30
Macera	2017	5	13		0.385 (0.139, 0.684)	3.24
Preda	2017	4	11		0.364 (0.109, 0.692)	2.97
Subtotal ($I^2 = 0.000\%$, $P = 0.634$)					0.211 (0.158, 0.269)	36.17
IFN-based						
Liaw	1997	0	15		0.000 (0.000, 0.218)	3.46
Villa	2001	1	30		0.033 (0.001, 0.172)	4.53
Yalcin	2002	1	5		0.200 (0.005, 0.716)	1.86
Chuang	2005	14	42		0.333 (0.196, 0.495)	4.97
Saitta	2006	2	9		0.222 (0.028, 0.600)	2.66
Senturk	2008	0	36		0.000 (0.000, 0.097)	4.77
Potthoff	2008	4	19		0.211 (0.061, 0.456)	3.84
Yu	2009	11	50		0.220 (0.115, 0.360)	5.17
Erol	2009	2	6		0.333 (0.043, 0.777)	2.09
Liu	2009	28	161		0.174 (0.119, 0.241)	6.09
Vigano	2009	4	22		0.182 (0.052, 0.403)	4.07
Hung	2011	4	135		0.030 (0.008, 0.074)	6.00
Kim	2011	2	18		0.111 (0.014, 0.347)	3.75
Yeh	2015	18	203		0.089 (0.053, 0.137)	6.19
Aygen	2017	7	27		0.259 (0.111, 0.463)	4.37
Subtotal ($I^2 = 78.170\%$, $P = 0.000$)					0.119 (0.063, 0.186)	63.83
Heterogeneity between groups: $P = 0.020$					0.157 (0.106, 0.215)	100.00
Overall ($I^2 = 72.662\%$, $P = 0.000$);						

Proportion (%): 0   0.25   0.5   0.75

Figure 2 Hepatitis B virus reactivation in hepatitis C virus patients with chronic hepatitis B virus infection. Pooled estimates of hepatitis B virus reactivation in hepatitis C virus patients with chronic hepatitis B surface antigen-positive hepatitis B virus infection.

and included eight prospective cohort studies[14-21], twenty-seven retrospective cohort studies[22-48], three randomized controlled trials[49-51] and one case-control study[52]. A total of 3468 patients with a median age of 53 years, were enrolled, including 1060 HBsAg-positive patients and 2408 patients with previous HBV infection. Thirty-five of these studies reported the number of HBV reactivations, thirty-five studies reported the number of hepatitis flares, and thirty-six studies reported the HCV SVR rates. HCV SVR with DAA-based therapy was achieved in 202 of 203 HBsAg-positive patients and 486 of 507 HBsAg-negative but HBcAb-positive patients. The baseline characteristics are shown in supplementary Table 1.

All articles were assessed as being of fair to good quality. However, studies with a small number of chronic HBV infections may have a potential risk of bias. The small-number effect on the pooled effect size was tested by meta-regression. The quality assessment of the included studies is shown in supplementary Table 2.

HBV reactivation

The random effects pooled overall HBV reactivation rate was 15.7% (95%CI: 10.6-21.5) in HBsAg-positive patients receiving anti-HCV treatment with statistical heterogeneity among the studies ($I^2 = 72.6\%$; $P < 0.001$; Figure 2). Subgroup analysis was performed according to the treatment regimen. The HBV reactivation rate was higher in the DAA-treated group (21.1%, 95%CI: 15.8-26.9) than in the IFN-treated group (11.9%, 95%CI: 6.3-18.6; $P = 0.02$ for subgroup differences). The HBV reactivation risk was further analyzed based on the HBV DNA level. We found that the HBV reactivation rate in patients with undetectable HBV DNA did not differ from that in those with detectable HBV DNA receiving either DAA-based or IFN-based therapy [relative risk (RR) 1.26, 95%CI: 0.84-1.89; $P = 0.255$]. No significant heterogeneity was found among the studies ($I^2 = 32.4\%$; $P = 0.123$; Figure 3). Egger's test showed a certain publication bias ($P = 0.03$; Figure 4). Univariate meta-regression analysis of the HBV reactivation rate showed
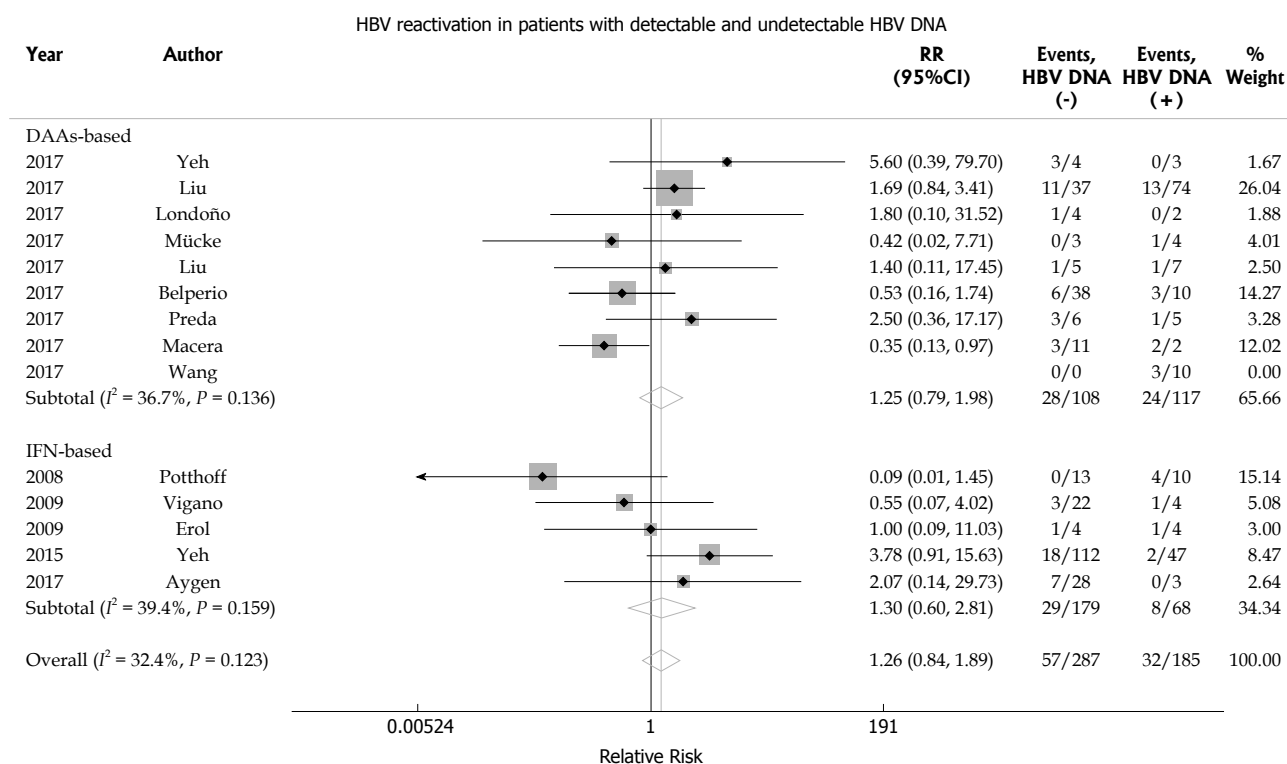
Figure 3 Hepatitis B virus reactivation according to baseline hepatitis B virus DNA level in hepatitis C virus patients with chronic hepatitis B virus infection. Relative risk of hepatitis B virus (HBV) reactivation according to baseline HBV DNA level in hepatitis C virus patients with chronic hepatitis B surface antigen-positive HBV infection.
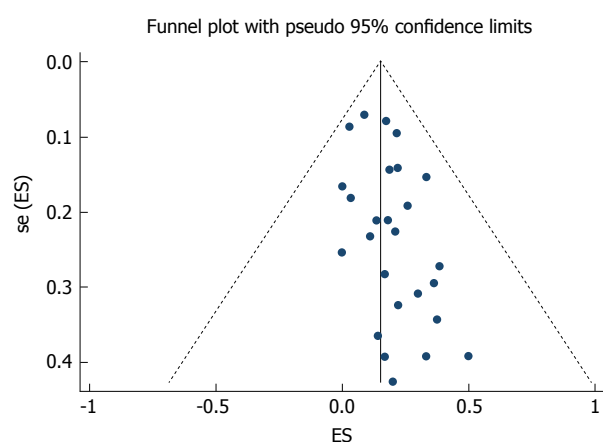

Figure 4 Funnel plot of 26 studies estimating the hepatitis B virus reactivation rates in hepatitis C virus patients with chronic hepatitis B virus infection. The vertical line corresponds to the summary hepatitis B virus reactivation rates as estimated from the random effect model. The publication bias is presented; *P* = 0.03, as tested by Egger's test.

that no baseline characteristics influenced the between-subgroup variation (all *P* > 0.05)

In patients with previous HBV infection, the HBV reactivation rate was 0.5% (95%CI: 0-1.3), with significant heterogeneity among the studies (I^2 = 61.5%; *P* = 0.001; Figure 5). In the subgroup analysis, the incidence of HBV reactivation was not different between the DAA-treated group (0.6%, 95%CI: 0-1.6) and the IFN-treated group (0, 95%CI: 0-1.1; *P* = 0.241 for subgroup differences). No significant publication bias was

found using Egger's test (*P* = 0.115).

HBV reactivation-related hepatitis
The incidence of hepatitis related to HBV reactivation was 0.7% (95%CI: 0-2.6) in HBsAg-positive patients with statistical heterogeneity among the studies (I^2 = 51.9%; *P* = 0.001; Figure 6). Subgroup analysis revealed that HBsAg-positive patients receiving DAA therapy (3.8%, 95%CI: 0.3-9.5) had a higher rate of HBV reactivation-related hepatitis than those receiving IFN-based therapy (0, 95%CI: 0-0.9; *P* = 0.007 for subgroup differences). Egger's test showed a significant publication bias (*P* = 0.015). None of the patients with previous HBV infection experienced hepatitis related to HBV reactivation.

According to baseline HBV DNA level, the rate of hepatitis was lower in patients with undetectable HBV DNA than in patients with detectable HBV DNA (RR = 0.39, 95%CI: 0.17-0.89; *P* = 0.025; Figure 7). Subgroup analysis revealed that the rate of hepatitis was lower in patients with undetectable HBV DNA than in patients with detectable HBV DNA in the DAA-treated group (RR = 0.20, 95%CI: 0.06-0.64; *P* = 0.007). In addition, the incidence of hepatitis was similar in IFN-treated patients with undetectable or detectable HBV DNA (RR = 1.10, 95%CI: 0.26-4.60; *P* = 0.895). No significant heterogeneity was found among the studies (I^2 = 0; *P* = 0.633).

HBV reactivation with and without antiviral prophylaxis
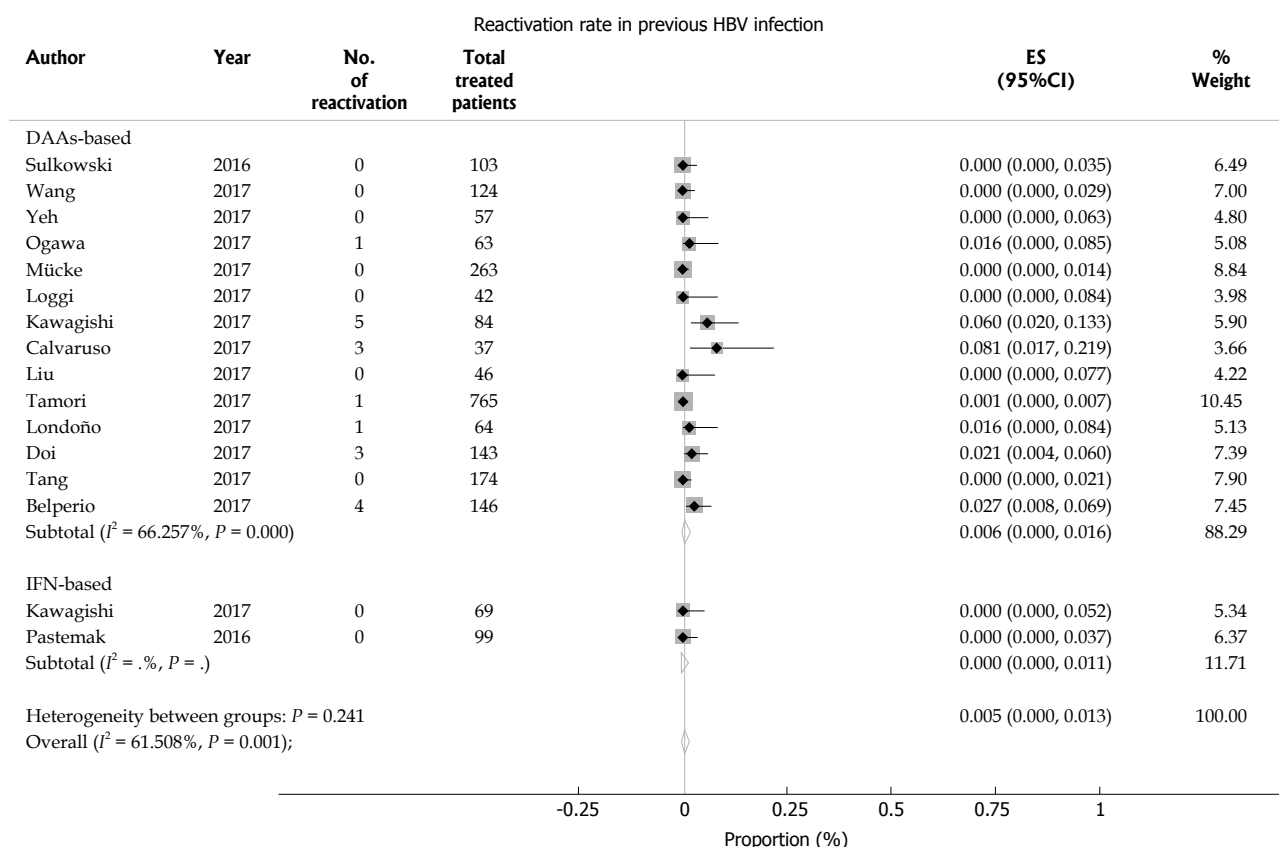We identified six studies that compared the HBV react-

Reactivation rate in previous HBV infection

Author	Year	No. of reactivation	Total treated patients		ES (95%CI)	% Weight
DAAs-based						
Sulkowski	2016	0	103		0.000 (0.000, 0.035)	6.49
Wang	2017	0	124		0.000 (0.000, 0.029)	7.00
Yeh	2017	0	57		0.000 (0.000, 0.063)	4.80
Ogawa	2017	1	63		0.016 (0.000, 0.085)	5.08
Mücke	2017	0	263		0.000 (0.000, 0.014)	8.84
Loggi	2017	0	42		0.000 (0.000, 0.084)	3.98
Kawagishi	2017	5	84		0.060 (0.020, 0.133)	5.90
Calvaruso	2017	3	37		0.081 (0.017, 0.219)	3.66
Liu	2017	0	46		0.000 (0.000, 0.077)	4.22
Tamori	2017	1	765		0.001 (0.000, 0.007)	10.45
Londoño	2017	1	64		0.016 (0.000, 0.084)	5.13
Doi	2017	3	143		0.021 (0.004, 0.060)	7.39
Tang	2017	0	174		0.000 (0.000, 0.021)	7.90
Belperio	2017	4	146		0.027 (0.008, 0.069)	7.45
Subtotal (I^2 = 66.257%, P = 0.000)					0.006 (0.000, 0.016)	88.29
IFN-based						
Kawagishi	2017	0	69		0.000 (0.000, 0.052)	5.34
Pastemak	2016	0	99		0.000 (0.000, 0.037)	6.37
Subtotal (I^2 = .%, P = .)					0.000 (0.000, 0.011)	11.71
Heterogeneity between groups: P = 0.241					0.005 (0.000, 0.013)	100.00
Overall (I^2 = 61.508%, P = 0.001);						

```
        -0.25        0      0.25     0.5     0.75      1
                      Proportion (%)
```

Figure 5 Hepatitis B virus reactivation in hepatitis C virus patients with previous hepatitis B virus infection. Pooled estimates of hepatitis B virus (HBV) reactivation in hepatitis C virus patients with previous hepatitis B surface antigen-negative but HBcAb-positive HBV infection.

ivation rate in HBsAg-positive patients with and without antiviral prophylaxis during DAA-based therapy; these studies included 39 patients who received preemptive anti-HBV treatment at baseline and 104 patients who did not receive preemptive anti-HBV therapy[16,17,22,23,36,41]. None of the 39 patients receiving entecavir or tenofovir developed HBV reactivation, but 21 of the 104 HBsAg-positive patients who did not receive antiviral prophylaxis developed HBV reactivation and five patients developed HBV reactivation-related hepatitis while undergoing DAA therapy. This meta-analysis showed that preemptive anti-HBV therapy with entecavir or tenofovir significantly reduced the risk of HBV reactivation in patients receiving DAA-based treatment (RR = 0.31, 95%CI: 0.1-0.96; P = 0.042). No statistical heterogeneity was found among the included studies (I^2 = 0; P = 0.954; Figure 8).

DISCUSSION

Multiple studies have reported HBV reactivation in HCV/HBV coinfected patients treated with DAA, but the reactivation rate is unclear, and the clinical outcome can range from ALT flares to liver failure and even death[16]. We found that the HBV reactivation rate was higher in patients receiving DAA-based therapy than in those receiving IFN-based therapy. A previous study showed an HBV reactivation rate of 12.2% in DAA-treated patients, but only 18 patients treated with DAA were enrolled in the study, and the definition of HBV reactivation was vastly different[5]. The uniform definition of HBV reactivation according to the AASLD criteria was used in our study, and the HBV reactivation rate was higher than that reported by Chen *et al*[5]. Furthermore, our review showed that the rate of hepatitis was 3.8% in patients who received DAA-based therapy, whereas the incidence was 0 in patients who received IFN-based therapy. Although the HBV reactivation rate was not different between patients with undetectable or detectable serum HBV DNA at baseline, patients with detectable HBV DNA who received DAAs tended to have a higher proportion of HBV reactivation-related hepatitis. Wang *et al*[15] reported three patients who had clinically apparent HBV reactivation, and one with hepatic failure. Macera *et al*[16] reported five HBsAg-positive patients with hepatitis, two of whom developed hepatic failure despite rescue treatment with NUCs. In addition, the FDA reported three of twenty-nine patients with HBV reactivation who developed liver failure during treatment with DAAs[6]. HBV reactivation occurred more frequently and the clinical outcome was more severe in patients treated with DAAs. This effect may be explained as follows: HBV viral replication can be suppressed in patients with HCV infection and the rapid suppression of HCV viral load by DAA–treatment may create a permissive environment for HBV replication, resulting in HBV reactivation[53]. In addition, IFN exerts an antiviral effect to suppress HBV replication and delay the time to HBV reactivation[54], but DAAs do not have any effect on the innate antiviral
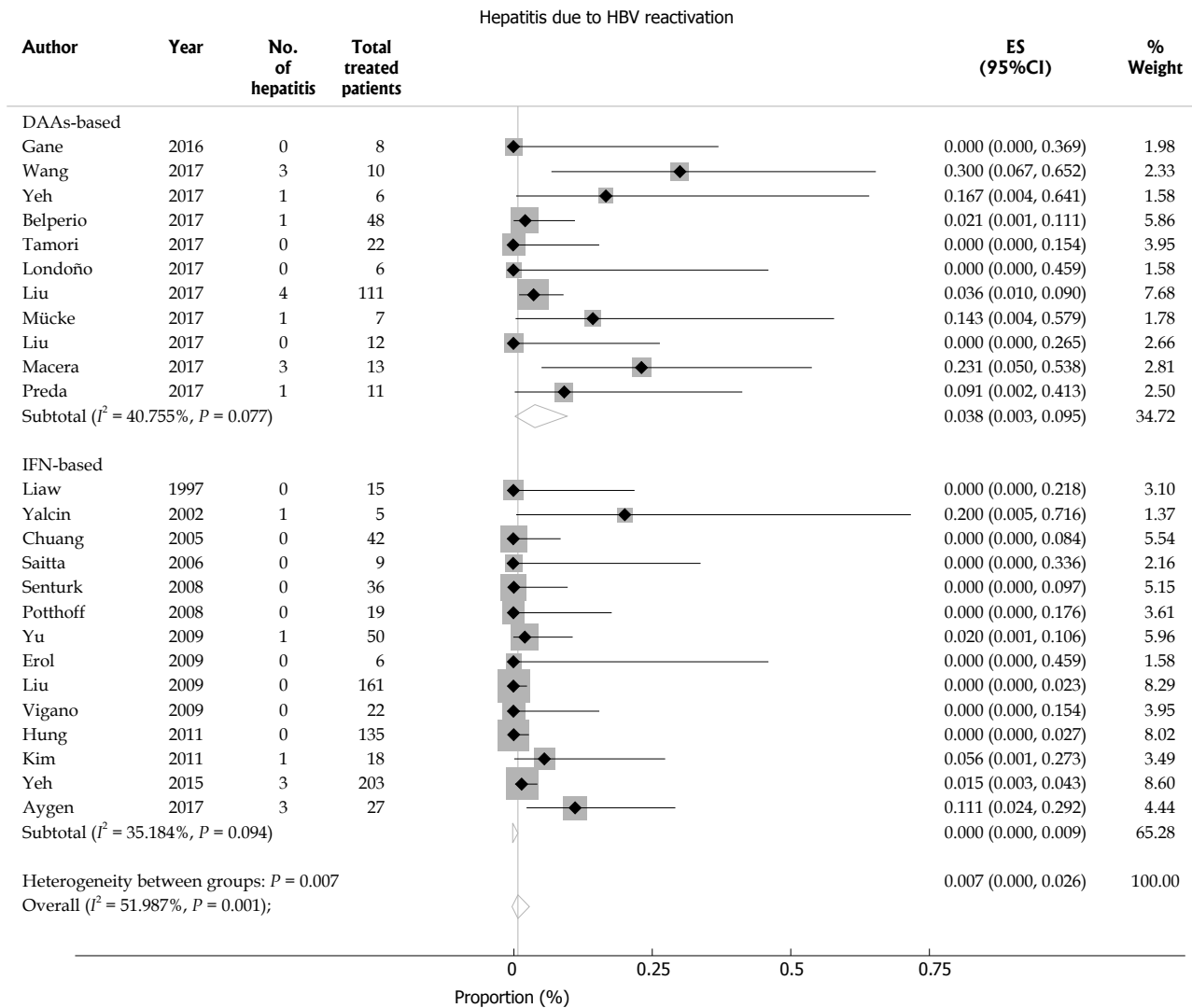
Hepatitis due to HBV reactivation

Author	Year	No. of hepatitis	Total treated patients		ES (95%CI)	% Weight
DAAs-based						
Gane	2016	0	8		0.000 (0.000, 0.369)	1.98
Wang	2017	3	10		0.300 (0.067, 0.652)	2.33
Yeh	2017	1	6		0.167 (0.004, 0.641)	1.58
Belperio	2017	1	48		0.021 (0.001, 0.111)	5.86
Tamori	2017	0	22		0.000 (0.000, 0.154)	3.95
Londoño	2017	0	6		0.000 (0.000, 0.459)	1.58
Liu	2017	4	111		0.036 (0.010, 0.090)	7.68
Mücke	2017	1	7		0.143 (0.004, 0.579)	1.78
Liu	2017	0	12		0.000 (0.000, 0.265)	2.66
Macera	2017	3	13		0.231 (0.050, 0.538)	2.81
Preda	2017	1	11		0.091 (0.002, 0.413)	2.50
Subtotal (I^2 = 40.755%, P = 0.077)					0.038 (0.003, 0.095)	34.72
IFN-based						
Liaw	1997	0	15		0.000 (0.000, 0.218)	3.10
Yalcin	2002	1	5		0.200 (0.005, 0.716)	1.37
Chuang	2005	0	42		0.000 (0.000, 0.084)	5.54
Saitta	2006	0	9		0.000 (0.000, 0.336)	2.16
Senturk	2008	0	36		0.000 (0.000, 0.097)	5.15
Potthoff	2008	0	19		0.000 (0.000, 0.176)	3.61
Yu	2009	1	50		0.020 (0.001, 0.106)	5.96
Erol	2009	0	6		0.000 (0.000, 0.459)	1.58
Liu	2009	0	161		0.000 (0.000, 0.023)	8.29
Vigano	2009	0	22		0.000 (0.000, 0.154)	3.95
Hung	2011	0	135		0.000 (0.000, 0.027)	8.02
Kim	2011	1	18		0.056 (0.001, 0.273)	3.49
Yeh	2015	3	203		0.015 (0.003, 0.043)	8.60
Aygen	2017	3	27		0.111 (0.024, 0.292)	4.44
Subtotal (I^2 = 35.184%, P = 0.094)					0.000 (0.000, 0.009)	65.28
Heterogeneity between groups: P = 0.007					0.007 (0.000, 0.026)	100.00
Overall (I^2 = 51.987%, P = 0.001);						

Proportion (%)

Figure 6 Hepatitis B virus reactivation-related hepatitis in hepatitis C virus patients with chronic hepatitis B virus infection. Overall risk of hepatitis B virus (HBV) reactivation-related hepatitis in hepatitis C virus patients with chronic hepatitis B surface antigen-positive HBV infection.

immune response.

Our meta-analysis revealed that preemptive anti-HBV therapy with NUCs significantly decreased the HBV reactivation rate. None of the patients receiving entecavir or tenofovir treatment experienced HBV reactivation while undergoing DAA therapy. The AASLD guideline recommends anti-HBV treatment for patients with active HBV infection[9]. However, the European Association for the Study of the Liver guideline recommends that HBsAg-positive patients treated with DAAs should receive concomitant antiviral prophylaxis at least 12 wk after DAA therapy[8]. Our study shows that preemptive anti-HBV treatment with NUCs is effective in preventing HBV reactivation in HBsAg-positive patients, especially in those with detectable HBV DNA who have a high risk of reactivation-related hepatitis.

We found that HBV reactivation was rare in patients with previous HBV infection, that the HBV reactivation rate was not different between patients receiving DAA-based treatment or IFN-based treatment, and that none of these patients experienced HBV reactivation-related hepatitis. Ogawa *et al*[34] reported that the anti-HBs titre may be associated with a risk of HBV reactivation, and that patients with previous HBV infection and negative anti-HBs or very low-titre anti-HBs are at risk of HBV reactivation. According to the results of our study, the HBV DNA level should be monitored during DAA therapy in patients with previous HBV infection, especially those with negative anti-HBs, even though the occurrence of HBV reactivation is rare in these patients.

There are several limitations in this meta-analysis. First, only four randomized controlled trials were included, and most of the studies were cohort studies with a lower data quality for analysis. Second, substantial heterogeneity was observed between the studies and may have caused overestimation or underestimation of the HBV reactivation risk. Third, there was an absence of unified assays for HBV DNA testing in several studies, especially in the IFN-treated group. In addition, we were unable to extract some detailed information that might have had an effect on HBV reactivation such as HBsAg level, anti-HBs titre, and HBV genotype. Further studies
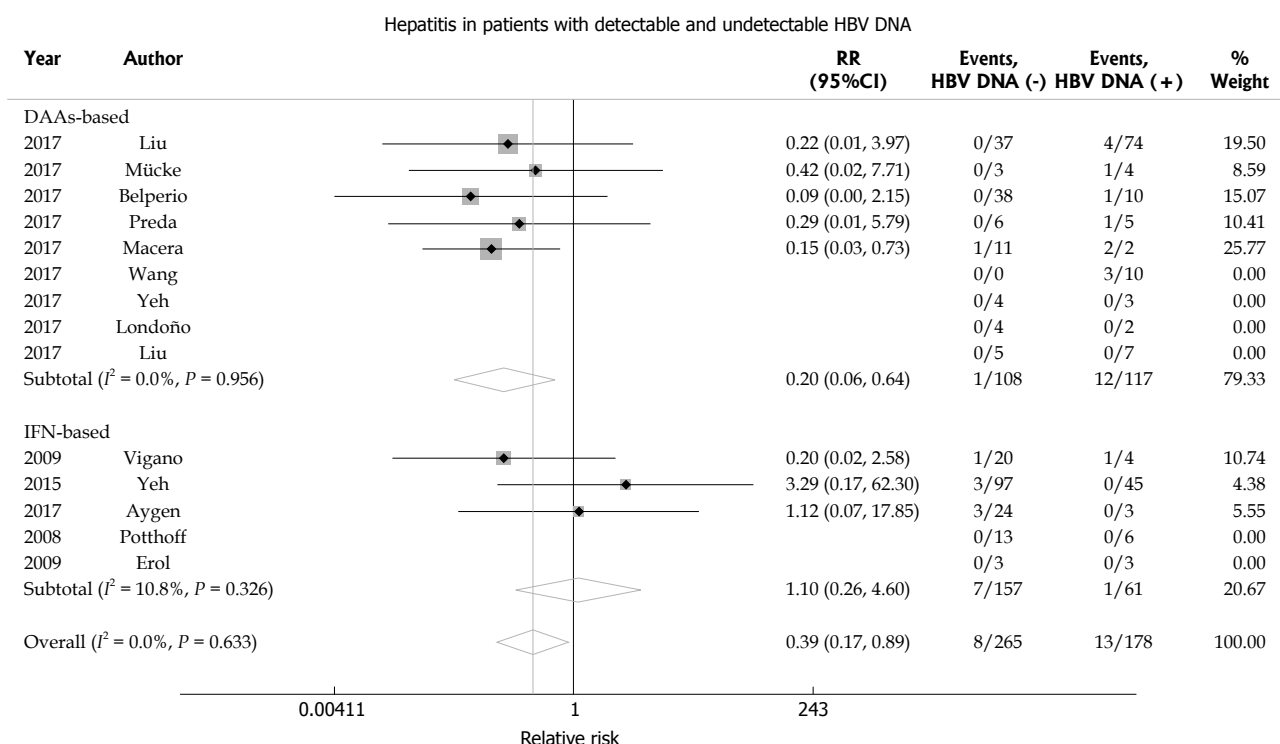
Figure 7 Hepatitis B virus reactivation-related hepatitis according to baseline hepatitis B virus DNA level. Relative risk of hepatitis B virus (HBV) reactivation-related hepatitis according to baseline HBV DNA level in hepatitis C virus patients with chronic hepatitis B surface antigen-positive HBV infection.
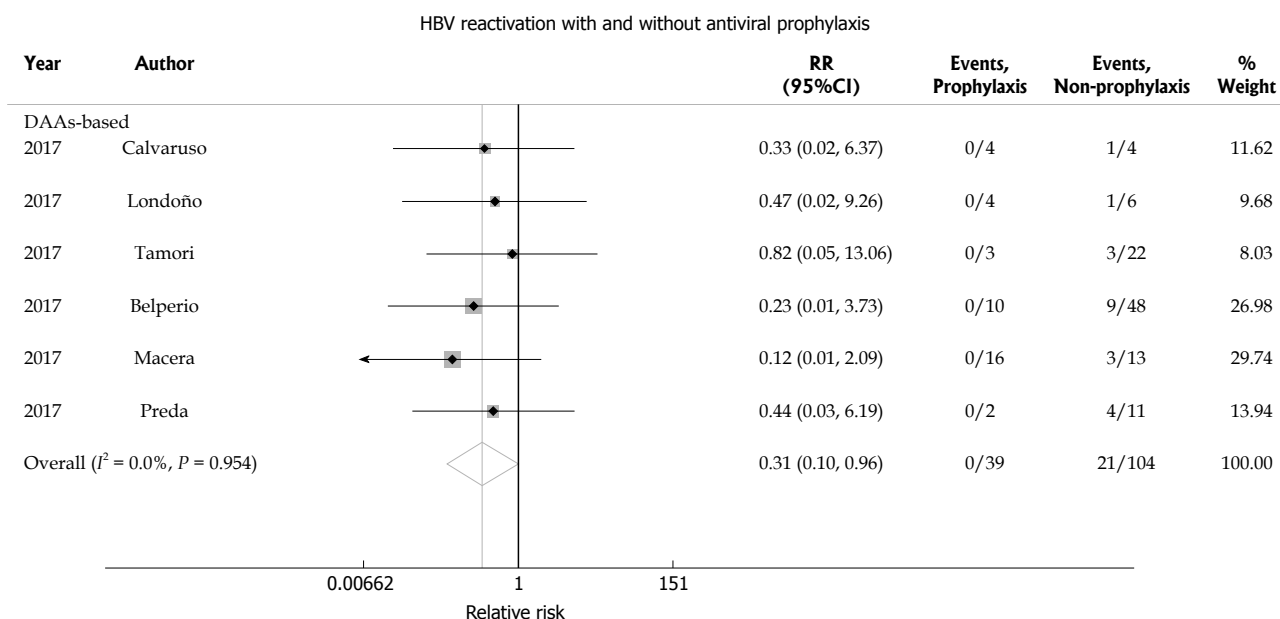

Figure 8 Hepatitis B virus reactivation in hepatitis B surface antigen-positive patients with and without antiviral prophylaxis. Relative risk of hepatitis B virus reactivation in hepatitis B surface antigen-positive patients with antiviral prophylaxis and without prophylaxis. HBV: Hepatitis B virus.

should be conducted to obtain relevant information about these parameters. Finally, the effect of the small number studies in our meta-analysis may have influenced the statistical accuracy of the study outcomes.

In conclusion, the present study found that HBV reactivation and hepatitis flare occurred more frequently in HBsAg-positive patients receiving DAA-based anti-HCV treatment than in patients receiving IFN-based

therapy. The incidence of HBV reactivation was rare in patients with previous HBV infection. In accordance with the guideline recommendations, our study showed that preemptive anti-HBV therapy with NUCs was effective in preventing HBV reactivation in HBsAg-positive patients, especially those with detectable serum HBV DNA. In patients with previous HBV infection receiving DAA treatment for HCV, especially those with negative

anti-HBs, the ALT level and HBV DNA level should be monitored. Further studies are required to assess the cost effectiveness of preemptive anti-HBV therapy in HBsAg-positive patients receiving DAA-based therapy.

ARTICLE HIGHLIGHTS

Research background
Hepatitis B virus/hepatitis C virus (HBV/HCV) dual infection is common in regions with high HBV prevalence, as HBV and HCV share a similar mode of transmission. HBV/HCV-coinfected patients tend to have more severe liver fibrosis and a higher risk of hepatocellular carcinoma than those without coinfection. HBV reactivation may occur after patients receive direct-acting antiviral agent (DAA)-based therapy or interferon (IFN)-based therapy for hepatitis C. Several studies have reported HBV reactivation but a meta-analysis to determine the proportion of HBV reactivation is still lacking.

Research motivation
Although previous studies reported HBV reactivation in patients undergoing DAA-based therapy or IFN-based therapy, the results indicate contradictory HBV reactivation rates. Moreover, the need for preemptive anti-HBV therapy remains controversial.

Research objectives
The main objectives of the systematic review and meta-analysis were to evaluate the incidence of HBV reactivation in patients receiving DAA-based therapy or IFN-based therapy for hepatitis C and the effectiveness of preemptive anti-HBV therapy for preventing HBV reactivation.

Research methods
Relevant publications were searched in the PubMed, MEDLINE and EMBASE databases with the indicated key words and subject terms. The data were extracted, and statistical analysis was conducted in Stata to assess the incidence of HBV reactivation and reactivation-related hepatitis. Significant heterogeneity was investigated using meta-regression and subgroup analyses for treatment regimen, HBV DNA level, study sample size, and race. Publication bias was tested using Egger's test and was assessed by funnel plots.

Research results
The systematic review identified 7092 articles and enrolled 39 full articles that met the inclusion criteria. The pooled random effects overall HBV reactivation rate was 15.7% (95%CI: 10.6-21.5) in hepatitis B surface antigen (HBsAg)-positive patients. The HBV reactivation rate was higher in the DAA-treated group (21.1%, 95%CI: 15.8-26.9) than in the IFN-treated group (11.9%, 95%CI: 6.3-18.6). The incidence of hepatitis related to HBV reactivation was 0.7% (95%CI: 0-2.6) in HBsAg-positive patients and patients receiving DAA therapy (3.8%, 95%CI: 0.3-9.5) had a higher rate of HBV reactivation-related hepatitis than those receiving IFN-based therapy (0, 95%CI: 0-0.9). Preemptive anti-HBV therapy with entecavir or tenofovir significantly reduced the risk of HBV reactivation in patients receiving DAA-based treatment (RR = 0.31, 95%CI: 0.1-0.96).

Research conclusions
Our study found that HBV reactivation and hepatitis flare occurred more frequently in HBsAg-positive patients receiving DAA-based anti-HCV treatment than in patients receiving IFN-based therapy. HBV reactivation was rare in patients with previous HBV infection. Preemptive anti-HBV therapy with NUCs was effective in HBsAg-positive patients to prevent HBV reactivation, especially in those with detectable serum HBV DNA.

Research perspectives
Our study found the high risk of HBV reactivation in patients receiving DAA-based therapy for hepatitis C. Preemptive anti-HBV treatment proved to be effective in preventing HBV reactivation during DAA therapy. However, more high quality randomized controlled trials are needed to assess the risk of HBV reactivation in patients with HBV/HCV coinfection. Further investigation

is required to assess the cost effectiveness of treating HBV/HCV-coinfected patients with NUCs prior to initiating DAA therapy.

REFERENCES

1 **Stanaway JD**, Flaxman AD, Naghavi M, Fitzmaurice C, Vos T, Abubakar I, Abu-Raddad LJ, Assadi R, Bhala N, Cowie B, Forouzanfour MH, Groeger J, Hanafiah KM, Jacobsen KH, James SL, MacLachlan J, Malekzadeh R, Martin NK, Mokdad AA, Mokdad AH, Murray CJL, Plass D, Rana S, Rein DB, Richardus JH, Sanabria J, Saylan M, Shahraz S, So S, Vlassov VV, Weiderpass E, Wiersma ST, Younis M, Yu C, El Sayed Zaki M, Cooke GS. The global burden of viral hepatitis from 1990 to 2013: findings from the Global Burden of Disease Study 2013. *Lancet* 2016; **388**: 1081-1088 [PMID: 27394647 DOI: 10.1016/S0140-6736(16)30579-7]

2 **Liu CJ**, Chen PJ, Chen DS. Dual chronic hepatitis B virus and hepatitis C virus infection. *Hepatol Int* 2009; **3**: 517-525 [PMID: 19669238 DOI: 10.1007/s12072-009-9147-9]

3 **Lee LP**, Dai CY, Chuang WL, Chang WY, Hou NJ, Hsieh MY, Lin ZY, Chen SC, Hsieh MY, Wang LY, Chen TJ, Yu ML. Comparison of liver histopathology between chronic hepatitis C patients and chronic hepatitis B and C-coinfected patients. *J Gastroenterol Hepatol* 2007; **22**: 515-517 [PMID: 17376043 DOI: 10.1111/j.1440-1746.2006.04547.x]

4 **Di Bisceglie AM**, Lok AS, Martin P, Terrault N, Perrillo RP, Hoofnagle JH. Recent US Food and Drug Administration warnings on hepatitis B reactivation with immune-suppressing and anticancer drugs: just the tip of the iceberg? *Hepatology* 2015; **61**: 703-711 [PMID: 25412906 DOI: 10.1002/hep.27609]

5 **Chen G**, Wang C, Chen J, Ji D, Wang Y, Wu V, Karlberg J, Lau G. Hepatitis B reactivation in hepatitis B and C coinfected patients treated with antiviral agents: A systematic review and meta-analysis. *Hepatology* 2017; **66**: 13-26 [PMID: 28195337 DOI: 10.1002/hep.29109]

6 **Bersoff-Matcha SJ**, Cao K, Jason M, Ajao A, Jones SC, Meyer T, Brinker A. Hepatitis B Virus Reactivation Associated With Direct-Acting Antiviral Therapy for Chronic Hepatitis C Virus: A Review of Cases Reported to the U.S. Food and Drug Administration Adverse Event Reporting System. *Ann Intern Med* 2017; **166**: 792-798 [PMID: 28437794 DOI: 10.7326/M17-0377]

7 **Pockros PJ**. Black Box Warning for Possible HBV Reactivation During DAA Therapy for Chronic HCV Infection. *Gastroenterol Hepatol* (NY) 2017; **13**: 536-540 [PMID: 29038644]

8 **European Association for the Study of the Liver**. EASL Recommendations on Treatment of Hepatitis C 2016. *J Hepatol* 2017; **66**: 153-194 [PMID: 27667367 DOI: 10.1016/j.jhep.2016.09.001]

9 American Association for the Study of Liver Disease, Infectious Diseases Society of America. Recommendations for testing, managing, and treating hepatitis C, 2016. Available from: URL: http://hcvguidelines.org/sites/default/files/HCV-Guidance_October_2016_a.pdf

10 **Liberati A**, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JP, Clarke M, Devereaux PJ, Kleijnen J, Moher D. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. *Ann Intern Med* 2009; **151**: W65-W94 [PMID: 19622512 DOI: 10.7326/0003-4819-151-4-200908180-00136]

11 **Hwang JP**, Lok AS. Management of patients with hepatitis B who require immunosuppressive therapy. *Nat Rev Gastroenterol Hepatol* 2014; **11**: 209-219 [PMID: 24247262 DOI: 10.1038/nrgastro.2013.216]

12 **Brewer JD**, Elston DM, Vidimos AT, Rizza SA, Miller SJ. Managing sharps injuries and other occupational exposures to HIV, HBV, and HCV in the dermatology office. *J Am Acad Dermatol* 2017; **77**: 946-951.e6 [PMID: 28865865 DOI: 10.1016/j.jaad.2017.06.040]

13 **Nyaga VN**, Arbyn M, Aerts M. Metaprop: a Stata command to perform meta-analysis of binomial data. *Arch Public Health* 2014; **72**: 39 [PMID: 25810908 DOI: 10.1186/2049-3258-72-39]

14 **Liu J**. Hepatitis B reactivation in chronic hepatitis C patients treated

with interferon or pan-oral direct-acting antivirals. *Hepatology* 2018;
67: 453-454 [PMID: 29080221 DOI: 10.1002/hep.29620]

15 **Wang C**, Ji D, Chen J, Shao Q, Li B, Liu J, Wu V, Wong A, Wang Y,
Zhang X, Lu L, Wong C, Tsang S, Zhang Z, Sun J, Hou J, Chen G,
Lau G. Hepatitis due to Reactivation of Hepatitis B Virus in Endemic
Areas Among Patients With Hepatitis C Treated With Direct-acting
Antiviral Agents. *Clin Gastroenterol Hepatol* 2017; **15**: 132-136
[PMID: 27392759 DOI: 10.1016/j.cgh.2016.06.023]

16 **Macera M**, Stanzione M, Messina V, D'Adamo G, Sangiovanni V,
Mioglioresi L, Fontanella L, De Pascalis S, Stornaiuolo G, Galeota
Lanza A, Ascione T, Sagnelli E, Gentile I, Piai G, Gaeta GB, Coppola
N. Interferon-Free Regimens in Hepatitis B Surface Antigen/Anti-
Hepatitis C Virus Positive Patients: The Need to Control Hepatitis
B Virus Replication to Avoid Hepatitis B Virus Reactivation. *Clin
Gastroenterol Hepatol* 2017; **15**: 1800-1802 [PMID: 28552801 DOI:
10.1016/j.cgh.2017.05.032]

17 **Londoño MC**, Lens S, Mariño Z, Bonacci M, Ariza X, Broquetas
T, Pla A, Bartres C, Adriani MV, Rodríguez-Tajes S, Costa J,
Carrión JA, Pérez-Del-Pulgar S, Forns X. Hepatitis B reactivation
in patients with chronic hepatitis C undergoing anti-viral therapy
with an interferon-free regimen. *Aliment Pharmacol Ther* 2017; **45**:
1156-1161 [PMID: 28206681 DOI: 10.1111/apt.13985]

18 **Doi A**, Sakamori R, Tahata Y, Urabe A, Morishita N, Yamada R,
Furuta K, Kodama T, Hikita H, Yakushijin T, Ohkawa K, Kaneko A,
Imai Y, Tatsumi T, Takehara T. Frequency of, and factors associated
with, hepatitis B virus reactivation in hepatitis C patients treated with
all-oral direct-acting antivirals: Analysis of a Japanese prospective
cohort. *Hepatol Res* 2017; **47**: 1438-1444 [PMID: 28585404 DOI:
10.1111/hepr.12919]

19 **Gane EJ**, Hyland RH, An D, Svarovskaia ES, Brainard D,
McHutchison JG. Ledipasvir and sofosbuvir for HCV infection
in patients coinfected with HBV. *Antivir Ther* 2016; **21**: 605-609
[PMID: 27367295 DOI: 10.3851/IMP3066]

20 **Saitta C**, Pontisso P, Brunetto MR, Fargion S, Gaeta GB, Niro GA,
Picciotto A, Smedile A, Squadrito G, Raimondo G. Virological
profiles in hepatitis B virus/hepatitis C virus coinfected patients
under interferon plus ribavirin therapy. *Antivir Ther* 2006; **11**:
931-934 [PMID: 17302256]

21 **Villa E**, Grottola A, Buttafoco P, Colantoni A, Bagni A, Ferretti I,
Cremonini C, Bertani H, Manenti F. High doses of alpha-interferon
are required in chronic hepatitis due to coinfection with hepatitis
B virus and hepatitis C virus: long term results of a prospective
randomized trial. *Am J Gastroenterol* 2001; **96**: 2973-2977 [PMID:
11693335 DOI: 10.1016/S0002-9270(01)03232-4]

22 **Belperio PS**, Shahoumian TA, Mole LA, Backus LI. Evaluation
of hepatitis B reactivation among 62,920 veterans treated with oral
hepatitis C antivirals. *Hepatology* 2017; **66**: 27-36 [PMID: 28240789
DOI: 10.1002/hep.29135]

23 **Calvaruso V**, Ferraro D, Licata A, Bavetta MG, Petta S, Bronte F,
Colomba G, Craxì A, Di Marco V. HBV reactivation in patients with
HCV/HBV cirrhosis on treatment with direct-acting antivirals. *J Viral
Hepat* 2018; **25**: 72-79 [PMID: 28703895 DOI: 10.1111/jvh.12754]

24 **Aygen B**, Günalo, Yildiz O, Çelen MK, Akhan S, Barut S, Ayaz C.
Hepatitis B Virus and Hepatitis C Virus Co-infection: An Evaluation
of Eighty-Two Patients. *J Viral Hepat* 2017; **23**: 14-19 [DOI:
10.4274/vhd.75768]

25 **Guptan RC**, Thakur V, Raina V, Sarin SK. Alpha-interferon therapy
in chronic hepatitis due to active dual infection with hepatitis B
and C viruses. *J Gastroenterol Hepatol* 1999; **14**: 893-898 [PMID:
10535471 DOI: 10.1046/j.1440-1746.1999.01952.x]

26 **Hasegawa I**, Orito E, Tanaka Y, Hiroshima N, Sakakibara K,
Sakurai M, Suzuki S, Sugauchi F, Ohno T, Ueda R, Mizokami M.
Impact of occult hepatitis B virus infection on efficacy and prognosis
of interferon-alpha therapy for patients with chronic hepatitis C.
Liver Int 2005; **25**: 247-253 [PMID: 15780046 DOI: 10.1111/
j.1478-3231.2005.1096.x]

27 **Hung CH**, Lu SN, Wang JH, Hu TH, Chen CH, Huang CM, Lee
CM. Sustained HCV clearance by interferon-based therapy reduces
hepatocellular carcinoma in hepatitis B and C dually-infected
patients. *Antivir Ther* 2011; **16**: 959-968 [PMID: 22024511 DOI:
10.3851/IMP1842]

28 **Kawagishi N**, Suda G, Onozawa M, Kimura M, Maehara O,
Ohara M, Izumi T, Umemura M, Ito J, Nakai M, Sho T, Natsuizaka
M, Morikawa K, Ogawa K, Sakamoto N. Comparing the risk of
hepatitis B virus reactivation between direct-acting antiviral therapies
and interferon-based therapies for hepatitis C. *J Viral Hepat* 2017;
24: 1098-1106 [PMID: 28632923 DOI: 10.1111/jvh.12737]

29 **Kim YJ**, Lee JW, Kim YS, Jeong SH, Kim YS, Yim HJ, Kim BH,
Lee CK, Park CK, Park SH. Clinical features and treatment efficacy
of peginterferon alfa plus ribavirin in chronic hepatitis C patients
coinfected with hepatitis B virus. *Korean J Hepatol* 2011; **17**:
199-205 [PMID: 22102386 DOI: 10.3350/kjhep.2011.17.3.199]

30 **Liu CJ**, Chuang WL, Lee CM, Yu ML, Lu SN, Wu SS, Liao LY,
Chen CL, Kuo HT, Chao YC, Tung SY, Yang SS, Kao JH, Liu CH,
Su WW, Lin CL, Jeng YM, Chen PJ, Chen DS. Peginterferon alfa-
2a plus ribavirin for the treatment of dual chronic infection with
hepatitis B and C viruses. *Gastroenterology* 2009; **136**: 496-504.e3
[PMID: 19084016 DOI: 10.1053/j.gastro.2008.10.049]

31 **Loggi E**, Gitto S, Galli S, Minichiello M, Conti F, Grandini E,
Scuteri A, Vitale G, Di Donato R, Cursaro C, Furlini G, Andreone
P. Hepatitis B virus reactivation among hepatitis C patients treated
with direct-acting antiviral therapies in routine clinical practice.
J Clin Virol 2017; **93**: 66-70 [PMID: 28654775 DOI: 10.1016/
j.jcv.2017.05.021]

32 **Mücke VT**, Mücke MM, Peiffer KH, Weiler N, Welzel TM, Sarrazin
C, Zeuzem S, Berger A, Vermehren J. No evidence of hepatitis B
virus reactivation in patients with resolved infection treated with
direct-acting antivirals for hepatitis C in a large real-world cohort.
Aliment Pharmacol Ther 2017; **46**: 432-439 [PMID: 28627791 DOI:
10.1111/apt.14177]

33 **Myers RP**, Thibault V, Poynard T. The impact of prior hepatitis
B virus infection on liver histology and the response to interferon
therapy in chronic hepatitis C. *J Viral Hepat* 2003; **10**: 103-110
[PMID: 12614466 DOI: 10.1046/j.1365-2893.2003.00407.x]

34 **Ogawa E**, Furusyo N, Murata M, Toyoda K, Hayashi T, Ura K.
Potential risk of HBV reactivation in patients with resolved HBV
infection undergoing direct-acting antiviral treatment for HCV. *Liver
Int* 2018; **38**: 76-83 [PMID: 28618152 DOI: 10.1111/liv.13496]

35 **Potthoff A**, Wedemeyer H, Boecher WO, Berg T, Zeuzem S, Arnold
J, Spengler U, Gruengreiff K, Kaeser T, Schuchmann M, Bergk A,
Forestier N, Deterding K, Manns MP, Trautwein C; Hep-Net B/C
Co-infection Study Group. The HEP-NET B/C co-infection trial: A
prospective multicenter study to investigate the efficacy of pegylated
interferon-alpha2b and ribavirin in patients with HBV/HCV co-
infection. *J Hepatol* 2008; **49**: 688-694 [PMID: 18490077 DOI:
10.1016/j.jhep.2008.03.028]

36 **Preda C,** Popescu C, Constantinescu I, Manuc M, Tugui L, Voiosu
R, Ceausu E, Diculescu M, Oproiu A. Outcome of patients with
compensated liver cirrhosis with hepatitis B virus + hepatitis C
virus coinfection treated with paritaprevir/ombitasvir/ritonavir,
dasabuvir with ribavirin: a national cohort study. *J Hepatol* 2017; **66**:
S295-S296 [DOI: 10.1016/s0168-8278(17)30907-8]

37 **Senturk H**, Tahan V, Canbakan B, Uraz S, Ulger Y, Ozaras R,
Tabak F, Mert A, Ozbay G. Chronic hepatitis C responds poorly to
combination therapy in chronic hepatis B carriers. *Neth J Med* 2008;
66: 191-195 [PMID: 18490796]

38 **Serpil E,** Ozkurt Z, Ozbek A, Parlak M. Poor Response to Treatment
with Peg-IFN Containing Regimens in Patients Coinfected with
Hepatitis B and Hepatitis C Virus. *Hepat Mon* 2009; **9**: 224-228

39 **Sulkowski MS**, Chuang WL, Kao JH, Yang JC, Gao B, Brainard
DM, Han KH, Gane E. No Evidence of Reactivation of Hepatitis
B Virus Among Patients Treated With Ledipasvir-Sofosbuvir for
Hepatitis C Virus Infection. *Clin Infect Dis* 2016; **63**: 1202-1204
[PMID: 27486112 DOI: 10.1093/cid/ciw507]

40 **Szymanek-Pasternak A**, Simon KA, Serafińska S, Janocha-Litwin J,
Pazgan-Simon M, Madej G. The influence of anti-HBc status on the
sustained virological response rate in HCV-infected patients treated
with pegylated interferon alfa 2 and ribavirin. *Clin Exp Hepatol*
2016; **2**: 155-160 [PMID: 28856281 DOI: 10.5114/ceh.2016.63873]

41 **Tamori A**, Abiru S, Enomoto H, Kioka K, Korenaga M, Tani J,

Enomoto M, Sugiyama M, Masaki T, Kawada N, Yatsuhashi H, Nishiguchi S, Mizokami M. Low incidence of hepatitis B virus reactivation and subsequent hepatitis in patients with chronic hepatitis C receiving direct-acting antiviral therapy. *J Viral Hepat* 2018; **25**: 608-611 [PMID: 29194858 DOI: 10.1111/jvh.12840]

42   **Tang L,** Tolaymat M, Stonesifer E, Kottilil S, Wilson E. Absence of hepatitis B reactivation among veterans with serological evidence of previous hepatitis B infection receiving anti-hepatitis C direct acting antivirals. *J Hepatol* 2017; **66**: S251-S252 [DOI: 10.1016/s0168-8278(17)30811-5]

43   **Viganò M**, Aghemo A, Iavarone M, Rumi MG, Agnelli F, Lampertico P, Donato MF, Colombo M. The course of inactive hepatitis B in hepatitis-C-coinfected patients treated with interferon and ribavirin. *Antivir Ther* 2009; **14**: 789-796 [PMID: 19812441 DOI: 10.3851/IMP1284]

44   **Weltman MD**, Brotodihardjo A, Crewe EB, Farrell GC, Bilous M, Grierson JM, Liddle C. Coinfection with hepatitis B and C or B, C and delta viruses results in severe chronic liver disease and responds poorly to interferon-alpha treatment. *J Viral Hepat* 1995; **2**: 39-45 [PMID: 7493293 DOI: 10.1111/j.1365-2893.1995.tb00070.x]

45   **Yalcin K,** Degertekin H. A 12 Month Course of Combination Therapy with Interferon and Ribavirin in Chronic Hepatitis due to Coinfection with Hepatitis B and C Viruses. *Clin Drug Invest* 2002; **22**: 797-798[DOI: 10.2165/00044011-200222110-00009]

46   **Yeh ML**, Huang CF, Hsieh MH, Ko YM, Chen KY, Liu TW, Lin YH, Liang PC, Hsieh MY, Lin ZY, Chen SC, Huang CI, Huang JF, Kuo PL, Dai CY, Yu ML, Chuang WL. Reactivation of hepatitis B in patients of chronic hepatitis C with hepatitis B virus infection treated with direct acting antivirals. *J Gastroenterol Hepatol* 2017; **32**: 1754-1762 [PMID: 28230928 DOI: 10.1111/jgh.13771]

47   **Yu JW**, Sun LJ, Zhao YH, Kang P, Gao J, Li SC. Analysis of the efficacy of treatment with peginterferon alpha-2a and ribavirin in patients coinfected with hepatitis B virus and hepatitis C virus. *Liver Int* 2009; **29**: 1485-1493 [PMID: 19602134 DOI: 10.1111/j.1478-3231.2009.02080.x]

48   **Zignego AL**, Fontana R, Puliti S, Barbagli S, Monti M, Careccia G, Giannelli F, Giannini C, Buzzelli G, Brunetto MR, Bonino F, Gentilini P. Impaired response to alpha interferon in patients with an inapparent hepatitis B and hepatitis C virus coinfection. *Arch Virol* 1997; **142**: 535-544 [PMID: 9349299 DOI: 10.1007/s007050050099]

49   **Liu CJ**, Chuang WL, Sheen IS, Wang HY, Chen CY, Tseng KC, Chang TT, Massetto B, Yang JC, Yun C, Knox SJ, Osinusi A, Camus G, Jiang D, Brainard DM, McHutchison JG, Hu TH, Hsu YC, Lo GH, Chu CJ, Chen JJ, Peng CY, Chien RN, Chen PJ. Efficacy of Ledipasvir and Sofosbuvir Treatment of HCV Infection in Patients Coinfected With HBV. *Gastroenterology* 2018; **154**: 989-997 [PMID: 29174546 DOI: 10.1053/j.gastro.2017.11.011]

50   **Yeh ML**, Hsieh MY, Huang CI, Huang CF, Hsieh MH, Liang PC, Lin YH, Hou NJ, Lin ZY, Chen SC, Huang JF, Dai CY, Chuang WL, Yu ML. Personalized Therapy of Chronic Hepatitis C and B Dually Infected Patients With Pegylated Interferon Plus Ribavirin: A Randomized Study. *Medicine* (Baltimore) 2015; **94**: e1837 [PMID: 26496327 DOI: 10.1097/MD.0000000000001837]

51   **Liaw YF**, Chien RN, Lin SM, Yeh CT, Tsai SL, Sheen IS, Chu CM. Response of patients with dual hepatitis B virus and C virus infection to interferon therapy. *J Interferon Cytokine Res* 1997; **17**: 449-452 [PMID: 9282824 DOI: 10.1089/jir.1997.17.449]

52   **Chuang WL**, Dai CY, Chang WY, Lee LP, Lin ZY, Chen SC, Hsieh MY, Wang LY, Yu ML. Viral interaction and responses in chronic hepatitis C and B coinfected patients with interferon-alpha plus ribavirin combination therapy. *Antivir Ther* 2005; **10**: 125-133 [PMID: 15751770]

53   **Eyre NS**, Phillips RJ, Bowden S, Yip E, Dewar B, Locarnini SA, Beard MR. Hepatitis B virus and hepatitis C virus interaction in Huh-7 cells. *J Hepatol* 2009; **51**: 446-457 [PMID: 19596477 DOI: 10.1016/j.jhep.2009.04.025]

54   **Lau GK**, Piratvisuth T, Luo KX, Marcellin P, Thongsawat S, Cooksley G, Gane E, Fried MW, Chow WC, Paik SW, Chang WY, Berg T, Flisiak R, McCloud P, Pluck N; Peginterferon Alfa-2a HBeAg-Positive Chronic Hepatitis B Study Group. Peginterferon Alfa-2a, lamivudine, and the combination for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2005; **352**: 2682-2695 [PMID: 15987917 DOI: 10.1056/NEJMoa043470]

CASE REPORT

Regulating migration of esophageal stents - management using a Sengstaken-Blakemore tube: A case report and review of literature

Hisaho Sato, Kaoru Ishida, Shusaku Sasaki, Masahiro Kojika, Shigeatsu Endo, Yoshihiro Inoue, Akira Sasaki

Hisaho Sato, Masahiro Kojika, Shigeatsu Endo, Yoshihiro Inoue, Department of Critical Care Medicine, Iwate Medical University, School of Medicine, Morioka, Iwate 020-0023, Japan

Kaoru Ishida, Shusaku Sasaki, Masahiro Kojika, Akira Sasaki, Department of Surgery, Iwate Medical University, School of Medicine, Morioka, Iwate 020-0023, Japan

Shigeatsu Endo, Morioka Yuai Hospital, Morioka, Iwate 020-0834, Japan

ORCID number: Hisaho Sato (0000-0002-9974-9796); Kaoru Ishida (0000-0003-1211-4323); Shusaku Sasaki (0000-0002-5042-6531); Masahiro Kojika (0000-0002-2418-1526); Shigeatsu Endo (0000-0002-1113-2952); Yoshihiro Inoue (0000-0003-1381-0769); Akira Sasaki (0000-0002-1346-5312).

Author contributions: Sato H, Kojika M, Endo S and Inoue Y designed the report; Ishida K, Sasaki S collected the patient's clinical data; Sato H, Kojika M and Sasaki A wrote the paper.

Informed consent statement: The patients and their families were offered written explanations prior to the start of the treatment, and they provided consent.

Conflict-of-interest statement: All the authors have no conflicts of interest to declare.

CARE Checklist (2013) statement: The authors have read the CARE Checklist (2013), and the manuscript was prepared and revised according to the CARE Checklist (2013).

Manuscript source: Unsolicited Manuscript

Correspondence to: Masahiro Kojika, MD, Department of Surgery, Iwate Medical University, School of Medicine, Morioka, Iwate 020-0023, Japan. kojimasa@iwate-med.ac.jp
Telephone: +81-19-6515111
Fax: +81-19-6515151

Abstract

Stent migration, which causes issues in stent therapy for esophageal perforations, can counteract the therapeutic effects and lead to complications. Therefore, techniques to regulate stent migration are important and lead to effective stent therapy. Here, in these cases, we placed a removable fully covered self-expandable metallic stent (FSEMS) in a 52-year-old man with suture failure after surgery to treat Boerhaave syndrome, and in a 53-year-old man with a perforation in the lower esophagus due to acute esophageal necrosis. At the same time, we nasally inserted a Sengstaken-Blakemore tube (SBT), passing it through the stent lumen. By inflating a gastric balloon, the lower end of the stent was supported. When the stent migration was confirmed, the gastric balloon was lifted slightly toward the oral side to correct the stent migration. In this manner, the therapy was completed for these two patients. Using a FSEMS and SBT is a therapeutic method for correcting stent migration and regulating the complete migration of the stent into the stomach without the patient undergoing endoscopic rearrangement of the stent. It was effective for positioning a stent crossing the esophagogastric junction.

Core tip: Here, we report two cases of stent migrations that were managed using Sengstaken-Blakemore tubes, which prevented complete migration to the stomach. The correction of the stent position was easy, and did not require endoscopic guidance. By regulating the stents, nutrition management became possible through a naso-enteric feeding tube. Both patients were discharged after oral ingestion became possible.

INTRODUCTION

The efficacy of stent placement as a nonsurgical treatment for perforation and suture failures in benign and malignant esophageal diseases has been shown[1,2]. However, a major problem with stent placement is stent migration. Stents that cross the esophagogastric junction (EGJ) are 7.5 times more likely to migrate than stents that do not cross the EGJ[3]. Migration can cause complications and requires repositioning with endoscopic guidance, which are disadvantageous to the therapeutic course. Endoscopic sutures[4] and clipping[5] can reduce migration, but stent migration is difficult to prevent completely. The challenges in achieving the efficacy and therapeutic effects of a stent include sustainable placement at the perforated site and the prevention of stent migration to the stomach. We used a Sengstaken-Blakemore tube (SBT) to position a fully covered self-expandable metallic stent (FSEMS) crossing the EGJ for perforation of the distal esophagus, which enabled correction of stent migration without endoscopic rearrangement. This treatment proved effective for regulating complete migration of the stent into the stomach, so we are reporting our findings.

CASE REPORT

Case 1: Postoperative suture failure

A 52-year-old man with a history of alcoholic liver disease and gastric ulcer presented with complaints of chest pain after vomiting. Esophagogastroduodenoscopy (EGD) confirmed a rupture in the left wall of the lower esophagus, which led to a diagnosis of Boerhaave

syndrome. As the focus was confined within the mediastinum, nonsurgical management through fasting was selected. Two days into his hospitalization, leakage to the thoracic cavity due to a mediastinal perforation and changes in the respiratory and circulatory dynamics were confirmed; thus, surgery was selected. The perforated site was sutured, and a drain was placed *via* a left thoracotomy. Postoperatively, he exhibited fever with a body temperature of 40 ℃ or higher and respiratory condition exacerbation, which was confirmed to have led to suture failure, as observed on the contrast imaging (Figure 1A). Therefore, a FSEMS (Flexella-J; ELLA-CS., Ltd. Hradec Kralove, Czech Republic) of 110 mm in length and 23 mm in diameter and SBT (Sumitomo Bakelite Co., Ltd.) were inserted (Figure 1B). At the same time, a nasoenteric feeding tube was passed through the stent lumen and placed in the jejunum (Figure 2A). The bottom of the stent was supported by the SBT gastric balloon that was positioned in the stomach and inflated slightly larger than the stent diameter to prevent migration into the stomach (Figure 2B). During the course of this treatment, the stent migration was confirmed. While supporting the bottom (anal side) of the stent by inflating the SBT gastric balloon, the stent was lifted toward the oral side and repositioned (Figure 3A and B). After the stent placement, the fever began to decrease, along with decreasing discharge from the drain. The stent was removed 66 d after placement.

Case 2: Perforation due to acute esophageal necrosis

A 53-year-old man sought medical care in another hospital because of shock, with a systolic blood pressure of 7.9 kPa (60 mmHg). The patient could not accurately specify the site of his pain, and he was managed in the hospital's cardiovascular unit. The patient was transferred to our cardiovascular unit because the cause of shock remained unknown, even after he received a vasoactive agonist. A chest computed tomography (CT) scan revealed mediastinal emphysema and a left pneumothorax (Figure 4). The EGD confirmed black discoloration from the esophagus to the gastric junction (Figure 5A and B) and the site of the esophageal perforation (Figure 5C). In addition, the duodenum showed black discoloration (Figure 5D). His blood test result showed a creatinine level of 3.83 mg/dL. Anuria continued from his previous hospitalization, and an acute kidney injury was confirmed. With the administration of 80% oxygen, the partial of arterial oxygen/fraction of inspired oxygen ratio was 72 mmHg, and his respiratory and circulatory dynamics were unstable. A thoracic cavity drain was placed, and a FSEMS (Hanaro stent; M.I. Tech Co.Ltd) of 150 mm in length and 24 mm in diameter and SBT were inserted to treat the esophageal perforation. At the same time, respiratory management using a high-flow nasal cannula, and circulatory management through fluid resuscitation were started. A nasoenteric feeding tube was inserted through the stent lumen, and an inflated SBT gastric balloon was placed to regulate the
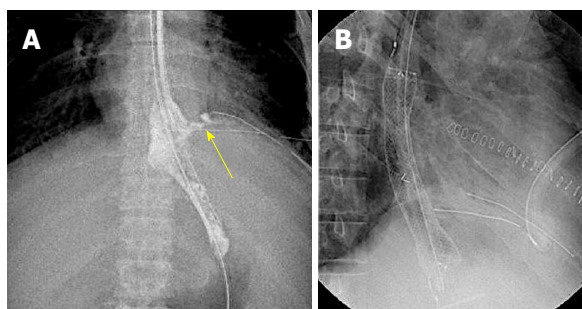
Figure 1 Fluoroscopic image. Leakage of the contrast agent from the suture site was confirmed (A, arrow); and a fully covered self-expandable metallic stent was placed in the leakage site (B).
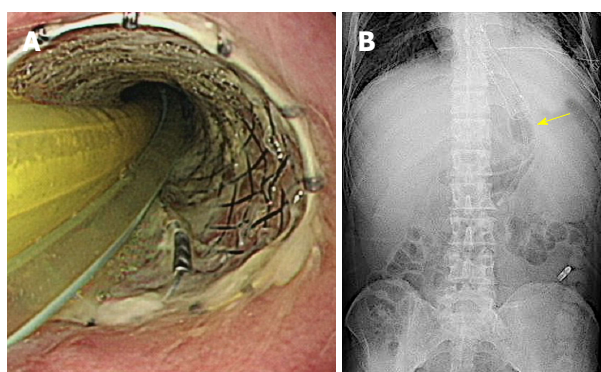

Figure 2 Sengstaken-Blakemore tube and nasoesophageal feeding tube placement. These tubes were placed by passing through the stent lumen (A); and the Sengstaken-Blakemore tube gastric balloon was inflated slightly larger than the stent diameter (B, arrow) to support the stent.
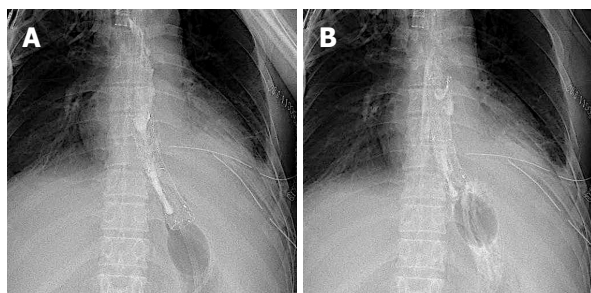

Figure 3 Fluoroscopic image. Stent migration was confirmed. While supporting the stent with the gastric balloon, the SBT was pulled toward the oral side to correct the position. A: Before repositioning; B: After repositioning. SBT: Sengstaken-Blakemore tube.

stent migration (Figure 6). Perforation closure (Figure 7A) and an improvement in the ischemic change in the duodenum (Figure 7B) were confirmed 38 d after the stent placement, and the stent was removed.

DISCUSSION

The incidence of esophageal perforation is rare, and the treatment experience at each facility is limited[6]. In recent years, the reports that support the usefulness of stent therapy for esophageal perforation and the reports on stent fixation have accumulated[7-10]. Stent therapy is

Figure 4 Chest computed tomography scan. Scan confirmed left pneumothorax (arrow) and mediastinal emphysema (arrow).

also recommended by a set of guidelines[11]. For effective stent placement, continuously covering the leakage from the perforated or suture site is a prerequisite. Stent migration is a serious issue that could counteract therapeutic effects and cause complications.

The incidence of stent migration is 18% to 40%[1,2,12,13]. However, stent migration in an esophageal perforation often involves malignant diseases, postoperative suture failure, Boerhaave syndrome, and iatrogenic causes, and it is difficult to evaluate the treatment for a specific cause or disease. In cases of benign diseases, the lack of stenosis to support a stent is the cause of stent migration, but when using a stent for hemostasis in esophageal variceal bleeding, migration has been confirmed in 21.6% of the cases[14]. As a therapeutic strategy against such stent migration, the effectiveness of a clip fixation[5] or migration prevention with silk threads has been shown[15]. However, stent migration is confirmed in 15.9% of the cases of SEMS fixed with endoscopic sutures[10] and in 13% of the cases in clip stent fixations[5]. When a stent migration is confirmed, the stent is often adjusted or repositioned endoscopically[1,8,12,13]; thus, removal or repositioning is required to the maximum extent allowed. Unfortunately, repetitive stent placements reduces the cost effectiveness[5]. Furthermore, stent migration away from the esophagus could lead to extraction due to migration to the stomach, intestinal obstruction due to migration to the small intestine[16], and injury to the spleen due to migration to the abdominal cavity[17].

The stents used in the present cases were FSEMS, but an FSEMS migrates more easily than a partially covered SEMS[6,8,11]. As the whole stent is covered, it easily slides against the esophageal mucosa. In addition, when readjusting the position of the stent with an SBT[18], we found that its slippery nature was an advantage. The initial insertion of the stent and SBT was performed with fluoroscopy, after administering a sedative. During hospitalization, the patients maintained normal levels of consciousness. Passing a SBT through the stent was simple, and no migration occurred through contact with the stent.

After inserting the SBT, a gastric balloon was inflated slightly larger than the diameter of the stent at the bot-
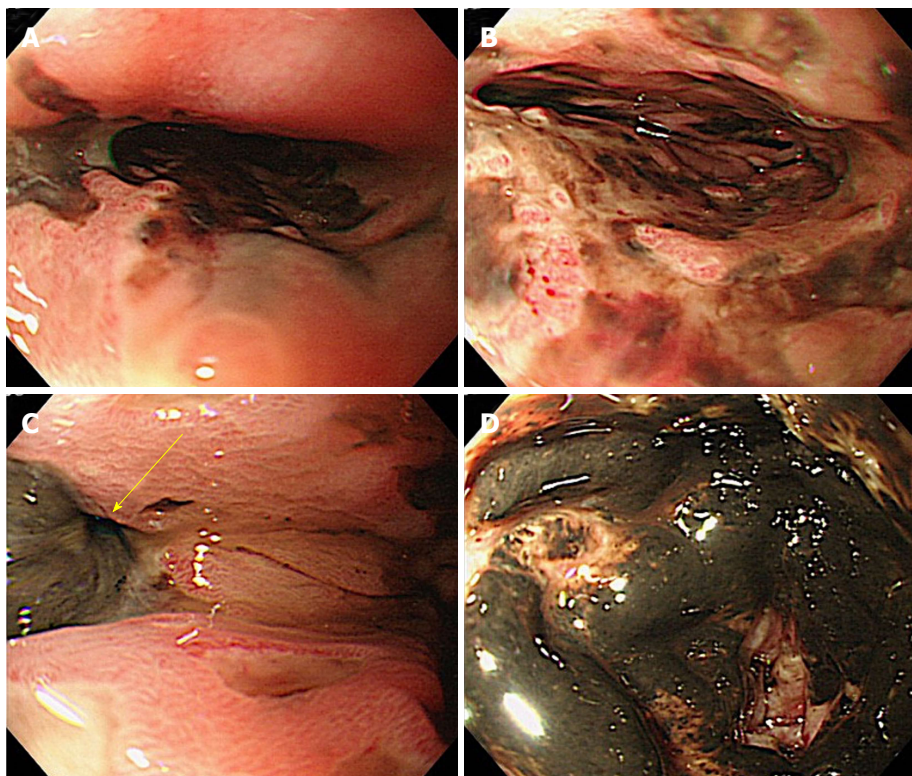
Figure 5 Initial Esophagogastroduodenoscopy findings. A and B: Black mucosal lesion was confirmed in the middle to lower esophagus; C: Perforation (arrow) due to necrosis was confirmed in the left wall of the lower esophagus; D: There was a black mucosal lesion in the duodenum.
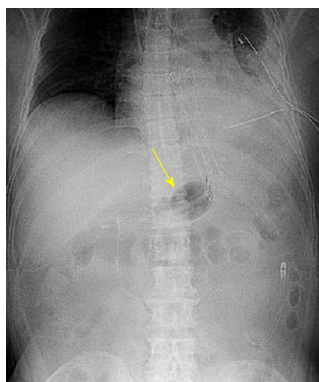

Figure 6 Abdominal radiograph. The SBT gastric balloon was inflated slightly larger than the stent diameter (arrow) to support the stent. The nasoesophageal feeding tube was positioned in the jejunum. SBT: Sengstaken-Blakemore tube.

tom to support and fix the stent in position. If the balloon is not inflated enough, it cannot retain the stent, but if it is inflated too much, it tends to be affected by gastric peristalsis. Thus, it is essential to check under fluoroscopy the amount of inflation that can retain the stent, which is slightly larger than the caliber of the bottom end of the stent, and fix the stent in place. SBT fixation was implemented by fixing with a nasal tape only, without traction. The gastric aspiration and esophageal openings decompressed the esophagus and stomach, and the stent migration was corrected by slowly pulling the SBT toward the oral side while keeping the gastric balloon inflated. The patient did experience

nasal discomfort but had no chest or abdominal pain. The stent migration was confirmed with a routine morning radiographic examination. Stent migration due to gastrointestinal peristalsis was found in the present treatment in which the stent movement was regulated by the SBT balloon. However, the migration from the leakage site was not complete because it was within the area where the stent therapy was still effective. When the migration was confirmed, complete migration to the stomach was prevented, and the optimum repair site for the leakage could be identified without using endoscopy. Overall, controlling a stent with an SBT is more cost-effective than repositioning with an endoscope. It should be noted that gastric balloons occasionally collapse naturally; thus, stent positions and gastric balloons must be checked under radiography. Before the treatment, we offered an explanation that we would be using a SBT, which is intended for hemostasis in esophageal variceal rupture. This is to regulate stent migration, however this incurs risks such as bleeding during repositioning and tissue damage, such as that caused by exacerbation of perforation. Treatment began after obtaining informed consent.

Nutrient pathways for patients with esophageal perforations, such as gastrostomy, jejunostomy, and non-oral feeding, are available[10]. In the present cases, the nutrients were administered by inserting a tube through the nose and positioning it in the jejunum. By passing the nasoenteric feeding tube through the stent lumen, the leakage site and nasoenteric feeding tube did not
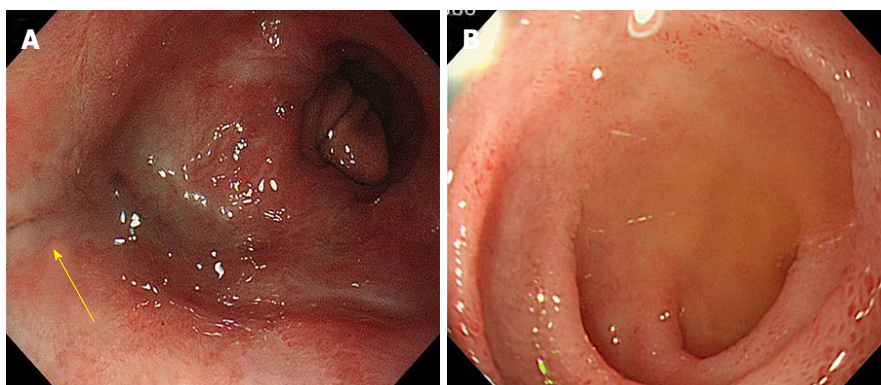
Figure 7 Esophagogastroduodenoscopy findings upon stent removal. A: Closure of the perforated site (arrow) was confirmed; B: The mucous membrane of the duodenum improved.

come in contact with each other; thus, there were no disadvantages. The balloon was not inflated immediately after insertion of the SBT, but rather after insertion of the nasoenteric feeding tube. No obstruction of the feeding tube due to compression by the balloon and stent, and no migration of the feeding tube due to the stent adjustment were observed.

The acute esophageal necrosis in Case 2 was a rare syndrome characterized by a diffuse black appearance around the circumference of the esophageal mucosa[19-22], which tends to develop in the distal third of the esophagus. Acute esophageal necrosis has been suggested to be caused by relatively low vascularity[23], and a duodenal lesion complication could develop at the same time. This compromises blood vessels diverging from the axis of the abdominal cavity, which provide the common blood supply to the distal part of the esophagus and duodenum[21,23,24]. In the present cases, although duodenal lesions were found, no complications such as bleeding caused by the nasoenteric feeding tube were observed. During the treatment with insertion of a SBT and nasoenteric feeding tube, no complication occurred other than an ulcer at the fixation site on the nose. The patient was discharged after oral ingestion was possible without transvenous or enteral nutrition, and without disturbance in the activities of daily living.

This case report has problems. First, with Case 1, if surgery and drainage had been selected as the initial treatment at admission, stent therapy may have been avoided. Second, closure of the postoperative suture leakage with clips may have been more effective than this treatment. Third, management using a stent and SBT may not be effective for proximal and central esophageal perforations. Lastly, this method does not completely prevent stent migration.

We herein provided SBT treatment for stent migration, which is the biggest problem in stent therapy. This treatment is effective for positioning stents that cross the EGJ in distal esophageal perforation. Early treatment is important for esophageal perforations, and stent therapy is not the only influence on prognosis. A multilateral treatment approach, including the appropriate management of breathing, circulation, infection, nutrition, drainage of the mediastinum, and thoracic cavity control, is necessary.

ARTICLE HIGHLIGHTS

Case characteristics
Using a fully covered self-expandable metallic stent (FSEMS) and Sengstaken-Blakemore tube (SBT) is a therapeutic method for correcting stent migration and regulating the complete migration of the stent into the stomach without the patient undergoing endoscopic rearrangement of the stent. It was effective for positioning a stent crossing the esophagogastric junction (EGJ).

Clinical diagnosis
The diagnoses were postoperative suture failure and perforation due to acute esophageal necrosis.

Differential diagnosis
When Case 2 was hospitalized, treatment was started under the suspicion of cardiovascular disease.

Laboratory diagnosis
In terms of the data before starting stent therapy, Case 1 had a white blood count of 7260 μL, CRP level of 11.8 mg/dL, and procalcitonin level of 1.5 ng/mL. *Pseudomonas* species were detected from the drain. Case 2 had a white blood cell count of 9850 μL, CRP level of 43.3 mg/dL, and procalcitonin level of 7.7 ng/mL. *Klebsiella* species were detected in the drain fluid sample.

Imaging diagnosis
Case 1 had contrast leakage from the suture site. Case 2 had a pneumothorax found on a computed tomography scan. An esophagogastroduodenoscopy revealed a blackened mucosal lesion, and perforations were found from the central to the lower part of the esophagus.

Pathological diagnosis
No pathological examination was performed.

Treatment
A FSEMS was used for the stent therapy, and stent migration was controlled using a SBT.

Related reports
No reports have described stent management using a SBT for esophageal

perforation.

Term explanation

SBTs are generally used to control bleeding from esophageal varices. Acute esophageal necrosis is a rare syndrome characterized by esophageal mucosa with a diffuse blackened appearance, and tends to occur in the distal one-third of the esophagus.

Experiences and lessons

This treatment is effective for positioning stents that cross the EGJ in distal esophageal perforation.

REFERENCES

1   **van Heel NC**, Haringsma J, Spaander MC, Bruno MJ, Kuipers EJ. Short-term esophageal stenting in the management of benign perforations. *Am J Gastroenterol* 2010; **105**: 1515-1520 [PMID: 20234349 DOI: 10.1038/ajg.2010.104]

2   **Gonzalez JM**, Servajean C, Aider B, Gasmi M, D'Journo XB, Leone M, Grimaud JC, Barthet M. Efficacy of the endoscopic management of postoperative fistulas of leakages after esophageal surgery for cancer: a retrospective series. *Surg Endosc* 2016; **30**: 4895-4903 [PMID: 26944730 DOI: 10.1007/s00464-016-4828-7]

3   **Eloubeidi MA**, Lopes TL. Novel removable internally fully covered self-expanding metal esophageal stent: feasibility, technique of removal, and tissue response in humans. *Am J Gastroenterol* 2009; **104**: 1374-1381 [PMID: 19491851 DOI: 10.1038/ajg.2009.133]

4   **Kantsevoy SV**, Bitner M. Esophageal stent fixation with endoscopic suturing device (with video). *Gastrointest Endosc* 2012; **76**: 1251-1255 [PMID: 23031249 DOI: 10.1016/j.gie.2012.08.003]

5   **Vanbiervliet G**, Filippi J, Karimdjee BS, Venissac N, Iannelli A, Rahili A, Benizri E, Pop D, Staccini P, Tran A, Schneider S, Mouroux J, Gugenheim J, Benchimol D, Hébuterne X. The role of clips in preventing migration of fully covered metallic esophageal stents: a pilot comparative study. *Surg Endosc* 2012; **26**: 53-59 [PMID: 21792721 DOI: 10.1007/s00464-011-1827-6]

6   **Biancari F**, D'Andrea V, Paone R, Di Marco C, Savino G, Koivukangas V, Saarnio J, Lucenteforte E. Current treatment and outcome of esophageal perforations in adults: systematic review and meta-analysis of 75 studies. *World J Surg* 2013; **37**: 1051-1059 [PMID: 23440483 DOI: 10.1007/s00268-013-1951-7]

7   **Sharma P**, Kozarek R; Practice Parameters Committee of American College of Gastroenterology. Role of esophageal stents in benign and malignant diseases. *Am J Gastroenterol* 2010; **105**: 258-273; quiz 274 [PMID: 20029413 DOI: 10.1038/ajg.2009.684]

8   **van Boeckel PG**, Sijbring A, Vleggaar FP, Siersema PD. Systematic review: temporary stent placement for benign rupture or anastomotic leak of the oesophagus. *Aliment Pharmacol Ther* 2011; **33**: 1292-1301 [PMID: 21517921 DOI: 10.1111/j.1365-2036.2011.04663.x]

9   **Dasari BV**, Neely D, Kennedy A, Spence G, Rice P, Mackle E, Epanomeritakis E. The role of esophageal stents in the management of esophageal anastomotic leaks and benign esophageal perforations. *Ann Surg* 2014; **259**: 852-860 [PMID: 24509201 DOI: 10.1097/SLA.0000000000000564]

10 **Law R**, Prabhu A, Fujii-Lau L, Shannon C, Singh S. Stent migration following endoscopic suture fixation of esophageal self-expandable metal stents: a systematic review and meta-analysis.

Surg Endosc 2018; **32**: 675-681 [PMID: 28726147 DOI: 10.1007/s00464-017-5720-9]

11 **Spaander MC**, Baron TH, Siersema PD, Fuccio L, Schumacher B, Escorsell À, Garcia-Pagán JC, Dumonceau JM, Conio M, de Ceglie A, Skowronek J, Nordsmark M, Seufferlein T, Van Gossum A, Hassan C, Repici A, Bruno MJ. Esophageal stenting for benign and malignant disease: European Society of Gastrointestinal Endoscopy (ESGE) Clinical Guideline. *Endoscopy* 2016; **48**: 939-948 [PMID: 27626318 DOI: 10.1055/s-0042-114210]

12 **Aryaie AH**, Singer JL, Fayezizadeh M, Lash J, Marks JM. Efficacy of endoscopic management of leak after foregut surgery with endoscopic covered self-expanding metal stents (SEMS). *Surg Endosc* 2017; **31**: 612-617 [PMID: 27317034 DOI: 10.1007/s00464-016-5005-8]

13 **Freeman RK**, Van Woerkom JM, Ascioti AJ. Esophageal stent placement for the treatment of iatrogenic intrathoracic esophageal perforation. *Ann Thorac Surg* 2007; **83**: 2003-7; discussion 2007-8 [PMID: 17532387 DOI: 10.1016/j.athoracsur.2007.02.025]

14 **Shao XD**, Qi XS, Guo XZ. Esophageal Stent for Refractory Variceal Bleeding: A Systemic Review and Meta-Analysis. *Biomed Res Int* 2016; **2016**: 4054513 [PMID: 27517043 DOI: 10.1155/2016/4054513]

15 **Shim CS**, Cho YD, Moon JH, Kim JO, Cho JY, Kim YS, Lee JS, Lee MS. Fixation of a modified covered esophageal stent: its clinical usefulness for preventing stent migration. *Endoscopy* 2001; **33**: 843-848 [PMID: 11571679 DOI: 10.1055/s-2001-17326]

16 **Sabharwal T**, Gulati MS, Fotiadis N, Dourado R, Botha A, Mason R, Adam A. Randomised comparison of the FerX Ella antireflux stent and the ultraflex stent: proton pump inhibitor combination for prevention of post-stent reflux in patients with esophageal carcinoma involving the esophago-gastric junction. *J Gastroenterol Hepatol* 2008; **23**: 723-728 [PMID: 18410607 DOI: 10.1111/j.1440-1746.2008.05396.x]

17 **Khan S**, George N, Tharian B. Extraluminal migration of an esophageal metal stent causing splenic injury. *Endoscopy* 2016; **48**: E326 [PMID: 27741526 DOI: 10.1055/s-0042-116817]

18 **Sengstaken rw**, Blakemore AH. Balloon tamponage for the control of hemorrhage from esophageal varices. *Ann Surg* 1950; **131**: 781-789 [PMID: 15411151 DOI: 10.1097/00000658-195005000-00017]

19 **Goldenberg SP**, Wain SL, Marignani P. Acute necrotizing esophagitis. *Gastroenterology* 1990; **98**: 493-496 [PMID: 2295407 DOI: 10.1016/0016-5085(90)90844-Q]

20 **Gurvits GE**, Shapsis A, Lau N, Gualtieri N, Robilotti JG. Acute esophageal necrosis: a rare syndrome. *J Gastroenterol* 2007; **42**: 29-38 [PMID: 17322991 DOI: 10.1007/s00535-006-1974-z]

21 **Gurvits GE**, Cherian K, Shami MN, Korabathina R, El-Nader EM, Rayapudi K, Gandolfo FJ, Alshumrany M, Patel H, Chowdhury DN, Tsiakos A. Black esophagus: new insights and multicenter international experience in 2014. *Dig Dis Sci* 2015; **60**: 444-453 [PMID: 25297468 DOI: 10.1007/s10620-014-3382-1]

22 **Burtally A**, Gregoire P. Acute esophageal necrosis and low-flow state. *Can J Gastroenterol* 2007; **21**: 245-247 [PMID: 17431514 DOI: 10.1155/2007/920716]

23 **Gurvits GE**. Black esophagus: acute esophageal necrosis syndrome. *World J Gastroenterol* 2010; **16**: 3219-3225 [PMID: 20614476 DOI: 10.3748/wjg.v16.i26.3219]

24 **Del Hierro PM**. Acute Necrotizing Esophagitis Followed by Duodenal Necrosis. *Gastroenterology Res* 2011; **4**: 286-288 [PMID: 27957030 DOI: 10.4021/gr361w]

Genetic analysis is helpful for the diagnosis of small bowel ulceration

Junji Umeno, Takayuki Matsumoto, Atsushi Hirano, Yuta Fuyuno, Motohiro Esaki

Junji Umeno, Atsushi Hirano, Yuta Fuyuno, Motohiro Esaki, Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Fukuoka 812-8582, Japan

Takayuki Matsumoto, Division of Gastroenterology, Department of Internal Medicine, Faculty of Medicine, Iwate Medical University, Morioka, Iwate 020-8505, Japan

Motohiro Esaki, Department of Endoscopic Diagnostics and Therapeutics, Saga University Hospital, Saga 849-8501, Japan

ORCID number: Junji Umeno (0000-0002-5253-5600); Takayuki Matsumoto (0000-0001-9786-3854); Atsushi Hirano (0000-0002-4791-4550); Yuta Fuyuno (0000-0003-3527-5648); Motohiro Esaki (0000-0002-1195-0074).

Correspondence to: Junji Umeno, MD, PhD, Doctor, Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan. jumeno@intmed2.med.kyushu-u.ac.jp
Telephone: +81-92-6425261
Fax: +81-92-6425273

Abstract

The widespread use of capsule endoscopy and balloon-assisted endoscopy has provided easy access for detailed mucosal assessment of the small intestine. However, the diagnosis of rare small bowel diseases, such as cryptogenic multifocal ulcerous stenosing enteritis (CMUSE), remains difficult because clinical and morphological features of these diseases are obscure even for gastroenterologists. In an issue of this journal in 2017, Hwang *et al* reviewed and summarized clinical and radiographic features of 20 patients with an established diagnosis of CMUSE. Recently, recessive mutations in the *PLA2G4A* and *SLCO2A1* genes have been shown to cause small intestinal diseases. The small bowel ulcers in each disease mimic those in the other and furthermore those found in nonsteroidal anti-inflammatory drug-induced enteropathy. These recent and novel findings suggest that a clinical diagnosis exclusively based on the characteristics of small bowel lesions is possibly imprecise. Genetic analyses seem to be inevitable for the diagnosis of rare small bowel disorders such as CMUSE.

Key words: Cryptogenic multifocal ulcerous stenosing enteritis; Chronic nonspecific multiple ulcers of the small intestine; Chronic enteropathy associated with *SLCO2A1* gene; Crohn's disease

Core tip: The purpose of this letter to the editor is to

comment on the differential diagnosis of small intestinal ulcers. Mutations in *PLA2G4A* and *SLCO2A1*, encoding proteins involved in the production and degradation of prostaglandins, cause rare gastrointestinal diseases with multiple small intestinal ulcers. In addition to conventional gastrointestinal examinations, genetic analyses are helpful in distinguishing these diseases.

TO THE EDITOR

We read with interest the article titled "Cryptogenic multifocal ulcerous stenosing enteritis: Radiologic features and clinical behavior" by Hwang *et al*[1]. This group reviewed the medical records of 36 patients suspected of having cryptogenic multifocal ulcerous stenosing enteritis (CMUSE) from seven hospitals in South Korea and finally diagnosed 20 patients as CMUSE. They performed a detailed investigation of clinical and radiographic features of the patients and claimed that radiologic features of CMUSE are multiple short strictures and/or shallow ulcers of the small intestine without significant bowel obstruction. They also stated that these radiologic features might be helpful in differentiating CMUSE from other inflammatory bowel diseases, especially Crohn's disease (CD).

Hwang *et al*[1] diagnosed CMUSE based on the published criteria[2,3]: (1) Unexplained small bowel strictures; (2) superficial ulcer in the mucosa and submucosa; (3) chronic or relapsing ulcerative stenosis and abdominal pain; (4) no signs of systemic inflammation; and (5) persistent and occult blood loss from the gastrointestinal tract except during bowel rest or the postoperative period. We agree with the diagnosis of the patients as non-CD, but we have a major concern about the diagnosis of CMUSE. First, they did not mention the history of nonsteroidal anti-inflammatory drug (NSAID) use, and the possibility of NSAID-induced enteropathy could not be excluded. Second, they did not distinguish CMUSE from chronic nonspecific multiple ulcers of the small intestine (CNSU). Recently, CMUSE has been found to be an autosomal recessive inherited disease caused by mutations in the *PLA2G4A* gene[4,5]. Because the *PLA2G4A* gene encodes cytoplasmic phospholipase A2-α (cPLA2α), which catalyzes the release of arachidonic acid from membrane phospholipids, CMUSE patients exhibit reduced production of prostaglandins and thromboxane A2, resulting in multiple ulcers of the small intestine and platelet dysfunction. We also identified that loss-of-function mutations in the *SLCO2A1* gene encoding a prostaglandin transporter cause CNSU and established a new disease entity as "chronic enteropathy associated

with *SLCO2A1* gene" (CEAS)[6]. Thus, additional genetic analysis is helpful in diagnosing CMUSE and CEAS. Given that the endoscopic and radiographic features of CMUSE, CEAS, and NSAID-induced enteropathy are quite similar[7], updated information on genetic tests and history of NSAID use is necessary to confirm the diagnosis. An accurate diagnosis also aids in further understanding the patient's clinical and radiographic features.

We have reported a nationwide survey with genetic analysis in Japanese patients with CEAS[8]. We believe that the prevalence rate of CEAS is increased compared with CMUSE because some pathogenic mutations of the *SLCO2A1* gene are observed in the general population according to the dbSNP database[9] (*e.g.*, the mutation allele frequency of rs765249238, c.940+1G>A is 0.00003295). By contrast, identified mutations of the *PLA2G4A* gene such as rs121434634 and rs121434635 are not observed in the general population. Therefore, it is possible that some patients in the study by Hwang *et al*[1] harbor recessive *SLCO2A1* gene mutations. The *SLCO2A1* gene encodes a prostaglandin transporter that mediates the uptake and clearance of prostaglandins. The *SLCO2A1* gene is also known as a cause of primary hypertrophic osteoarthropathy (PHO), which affects the skin and bones, presenting as digital clubbing, periostosis, acroosteolysis, painful joint enlargement, and thickened skin[10]. We previously reported that 30% of CEAS patients have at least one clinical feature of PHO as an extra-intestinal manifestation[8]. Data regarding the prevalence of these extra-intestinal manifestations among the patients are of particular interest. We are also eager to obtain this information for the patients in the study by Hwang *et al*[1].

In conclusion, additional genetic analysis should be helpful for the differential diagnosis of CMUSE and CEAS.

REFERENCES

1 **Hwang J**, Kim JS, Kim AY, Lim JS, Kim SH, Kim MJ, Kim MS, Song KD, Woo JY. Cryptogenic multifocal ulcerous stenosing enteritis: Radiologic features and clinical behavior. *World J Gastroenterol* 2017; **23**: 4615-4623 [PMID: 28740350 DOI: 10.3748/wjg.v23.i25.4615]

2 **Perlemuter G**, Guillevin L, Legman P, Weiss L, Couturier D, Chaussade S. Cryptogenetic multifocal ulcerous stenosing enteritis: an atypical type of vasculitis or a disease mimicking vasculitis. *Gut* 2001; **48**: 333-338 [PMID: 11171822 DOI: 10.1136/gut.48.3.333]

3 **Matsumoto T**, Iida M, Matsui T, Yao T. Chronic nonspecific multiple ulcers of the small intestine: a proposal of the entity from Japanese gastroenterologists to Western enteroscopists. *Gastrointest Endosc* 2007; **66**: S99-107 [PMID: 17709045 DOI: 10.1016/j.gie.2007.01.004]

4 **Adler DH**, Cogan JD, Phillips JA 3rd, Schnetz-Boutaud N, Milne GL, Iverson T, Stein JA, Brenner DA, Morrow JD, Boutaud O, Oates JA. Inherited human cPLA(2alpha) deficiency is associated with impaired eicosanoid biosynthesis, small intestinal ulceration, and platelet dysfunction. *J Clin Invest* 2008; **118**: 2121-2131 [PMID: 18451993 DOI: 10.1172/JCI30473]

5 **Brooke MA**, Longhurst HJ, Plagnol V, Kirkby NS, Mitchell JA, Rüschendorf F, Warner TD, Kelsell DP, MacDonald TT. Cryptogenic multifocal ulcerating stenosing enteritis associated with homozygous deletion mutations in cytosolic phospholipase A2-α. *Gut* 2014; **63**: 96-104 [PMID: 23268370 DOI: 10.1136/gutjnl-2012-303581]

6 **Umeno J**, Hisamatsu T, Esaki M, Hirano A, Kubokura N, Asano

K, Kochi S, Yanai S, Fuyuno Y, Shimamura K, Hosoe N, Ogata H, Watanabe T, Aoyagi K, Ooi H, Watanabe K, Yasukawa S, Hirai F, Matsui T, Iida M, Yao T, Hibi T, Kosaki K, Kanai T, Kitazono T, Matsumoto T. A Hereditary Enteropathy Caused by Mutations in the SLCO2A1 Gene, Encoding a Prostaglandin Transporter. *PLoS Genet* 2015; **11**: e1005581 [PMID: 26539716 DOI: 10.1371/journal.pgen.1005581]

7   **Esaki M**, Umeno J, Kitazono T, Matsumoto T. Clinicopathologic features of chronic nonspecific multiple ulcers of the small intestine. *Clin J Gastroenterol* 2015; **8**: 57-62 [PMID: 25788296 DOI: 10.1007/s12328-015-0559-x]

8   **Umeno J**, Esaki M, Hirano A, Fuyuno Y, Ohmiya N, Yasukawa S, Hirai F, Kochi S, Kurahara K, Yanai S, Uchida K, Hosomi S, Watanabe K, Hosoe N, Ogata H, Hisamatsu T, Nagayama M, Yamamoto H, Abukawa D, Kakuta F, Onodera K, Matsui T, Hibi T, Yao T, Kitazono T, Matsumoto T; CEAS study group. Clinical features of chronic enteropathy associated with SLCO2A1 gene: a new entity clinically distinct from Crohn's disease. *J Gastroenterol* 2018; **53**: 907-915 [PMID: 29313109 DOI: 10.1007/s00535-017-1426-y]

9   **Sherry ST**, Ward MH, Kholodov M, Baker J, Phan L, Smigielski EM, Sirotkin K. dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res* 2001; **29**: 308-311 [PMID: 11125122 DOI: 10.1093/nar/29.1.308]

10   **Zhang Z**, Xia W, He J, Zhang Z, Ke Y, Yue H, Wang C, Zhang H, Gu J, Hu W, Fu W, Hu Y, Li M, Liu Y. Exome sequencing identifies SLCO2A1 mutations as a cause of primary hypertrophic osteoarthropathy. *Am J Hum Genet* 2012; **90**: 125-132 [PMID: 22197487 DOI: 10.1016/j.ajhg.2011.11.019]

Baishideng®

World Journal of

Gastroenterology

Contents

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Rakesh Kumar Tandon, FRCP (Hon), MD, PhD, Doctor, Professor, Department of Gastroenterology, Pushpawati Singhania Research Institute for Liver, Renal and Digestive Diseases, Sheikh Sarai-Phase II, New Delhi 110017, Delhi, India

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 642 experts in gastroenterology and hepatology from 59 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

Upfront surgery of small intestinal neuroendocrine tumors. Time to reconsider?

Kosmas Daskalakis, Apostolos V Tsolakis

Kosmas Daskalakis, Department of Surgical Sciences, Uppsala University, Uppsala 75185, Sweden

Apostolos V Tsolakis, Department of Oncology and Pathology, Karolinska Institute, Stockholm SE-171 76, Sweden

Apostolos V Tsolakis, Cancer Center Karolinska, Karolinska University Hospital Solna R8:04, Stockholm SE-171 76, Sweden

Apostolos V Tsolakis, Department of Gastrointestinal Endoscopy, Karolinska University Hospital Huddinge, Stockholm SE-141 86, Sweden

ORCID number: Kosmas Daskalakis (0000-0003-4224-8912); Apostolos V Tsolakis (0000-0002-6784-5572).

Author contributions: Daskalakis K and Tsolakis AV both designed and wrote the editorial.

Abstract

Small intestinal neuroendocrine tumors (SI-NETs) may demonstrate a widely variable clinical behavior but usually it is indolent. In cases with localized disease, locoregional resective surgery (LRS) is generally indicated with a curative intent. LRS of SI-NETs is also the recommended treatment when symptoms are present, regardless of the disease stage. Concerning asymptomatic patients with distant metastases, prophylactic LRS has been traditionally suggested to avoid possible future complications. Even the current European Neuroendocrine Tumor Society guidelines emphasize a possible effect of LRS in Stage IV SI-NETs with unresectable liver metastases. On the contrary, the 2017 National Comprehensive Cancer Network Guidelines on carcinoid tumors do not support the resection of a small, asymptomatic, relatively stable primary tumor in the presence of unresectable metastatic disease. Furthermore, a recent study revealed no survival advantage for asymptomatic patients with distant-stage disease who underwent upfront LRS. At the aforementioned paper, it was suggested that delayed surgery as needed was comparable with the upfront surgical approach in terms of postoperative morbidity and mortality, the length of the hospital stay and the rate of incisional hernia repairs but was associated with fewer reoperations for bowel obstruction. On the other hand, it is also important to note that some patients might benefit from a prophylactic surgical approach and our attention should focus on identifying this patient population.

Key words: Small intestinal neuroendocrine tumors; Locoregional resective surgery

Core tip: Upfront locoregional resective surgery of small intestinal neuroendocrine tumors is the mainstay treatment when radical resection is feasible or when symptoms are present, regardless of the disease stage. However, in the light of contemporary evidence, the traditional upfront surgical approach is challenged regarding patients with distant metastases without local tumor-related symptoms.

INTRODUCTION

Small intestinal neuroendocrine tumors (SI-NETs) have an indolent clinical course and are often diagnosed at a late stage[1]. In patients without distant metastases, locoregional resective surgery (LRS) is generally indicated with a curative intent. However, in patients with distant-stage disease, LRS is generally not considered curative, although sometimes liver surgery or local ablative treatments are undertaken after or before radical LRS.

Even in the era of a broad panel of novel, targeted and systemic therapies for SI-NETs, recurrence after perceived radical liver resection is still very common, and neither liver resection nor radiofrequency ablation of liver metastases has unequivocally been found to prolong survival[2,3]. Therefore, even with the intention to achieve macroscopic radicality and cure, liver procedures for SI-NETs should generally be considered palliative[4].

STUDY ANALYSIS

Many patients with distant-stage disease may present with distinct clinical symptoms and signs due to hormonal excess and/or with local tumor-related symptoms causing abdominal pain, obstruction and/or an impaired blood supply to the intestines. These patients with local tumor-related symptoms generally undergo LRS at the time of diagnosis. Some patients may undergo an acute laparotomy because of an intestinal obstruction of unknown etiology. Others will undergo palliative surgery for a partial intestinal obstruction, bleeding, ischemic complications due to a tumor mass, or even for symptom relief in the cases of hormonal syndrome refractory to medical therapy.

The extension of mesenteric lymph node metastases below or above the horizontal part of the duodenum is a crucial factor for treatment since a number of patients will display mesenteric lymph node metastases in the root of the mesentery, with associated fibrosis, encasing the superior mesenteric vessels. These tumors are then usually considered inoperable. Palliative, minimally invasive measures such as stenting of the superior mesenteric vein have been applied to symptomatic patients with bulky mesenteric disease since LRS in these patients may be complicated and endanger circulation to substantial parts of the bowel[5].

Generally, for tumors originating in the proximal ileum and jejunum, segmental small intestinal resection is performed. However, for primary tumors located near the ileocecal valve in the distal ileum, ileocecal resection or right hemicolectomy is performed, with the latter possibly combined with improved clearance of regional lymph node metastases. Even though the latest Surveillance Epidemiology and End Results report challenges the prognostic significance of lymphatic metastasis for SI-NETs with locoregional disease only, there are certain biases and limitations in these data[6].

In asymptomatic patients with distant metastases, prophylactic LRS has been traditionally advocated to avoid a future intestinal obstruction, ischemia, perforation or bleeding. The survival rates of these patients after LRS, as reported in retrospective cohort studies, are probably largely influenced by both the selection bias and immortal time bias. Generally, there are differences in contemporary literature from up-to-date guidelines about approaching endocrine disorders[7]. The current ENETS guidelines emphasize a possible effect of LRS in Stage IV SI-NETs with unresectable liver metastases, but these guidelines are based on the information gathered from the abovementioned cohort studies[8,9].

On the other hand, the 2017 National Comprehensive Cancer Network Guidelines on carcinoid tumors advocate against resection of a small, asymptomatic, relatively stable primary tumor in the presence of unresectable metastatic disease[10]. A recent study has revealed no survival advantage for asymptomatic patients with distant-stage disease who underwent upfront LRS[11]. Interestingly, delayed surgery as needed was comparable with the upfront surgical approach in terms of postoperative morbidity and mortality, the length of the hospital stay and the rate of incisional hernia repairs but was associated with fewer reoperations for bowel obstruction[12]. These results are also consistent with the Rotterdam group findings that confirmed that there is no benefit of prophylactic surgery for overall survival[12]. However, it is also important to note that while patients with disseminated SI-NETs may not benefit from upfront prophylactic surgery, some patient populations might, *e.g.*, older patients or those with large tumors and patients with progressive locoregional disease[13]. Importantly, to be able to identify patients who might benefit from a prophylactic surgical approach, more insight is needed into the development of mesenteric fibrosis in SI-NETs[9].

PERSPECTIVE

In conclusion, LRS retains its value in the treatment of patients with SI-NETs when radical resection is feasible

or symptomatic disease is present, regardless of the disease stage.

However, current evidence challenges the traditional view that extensive LRS needs to be performed in patients with distant metastases in the absence of local tumor-related symptoms. A more conservative approach, with delayed LRS as clinically indicated, may be reasonable for the subset of asymptomatic SI-NET patients with distant-stage disease. This revised approach may complete the armamentarium of systemic and liver-directed treatments as indicated per patient.

REFERENCES

1   **Norlén O**, Stålberg P, Öberg K, Eriksson J, Hedberg J, Hessman O, Janson ET, Hellman P, Åkerström G. Long-term results of surgery for small intestinal neuroendocrine tumors at a tertiary referral center. *World J Surg* 2012; **36**: 1419-1431 [PMID: 21984144 DOI: 10.1007/s00268-011-1296-z]

2   **Elias D**, Lefevre JH, Duvillard P, Goéré D, Dromain C, Dumont F, Baudin E. Hepatic metastases from neuroendocrine tumors with a "thin slice" pathological examination: they are many more than you think. *Ann Surg* 2010; **251**: 307-310 [PMID: 20010089 DOI: 10.1097/SLA.0b013e3181bdf8cf]

3   **Sarmiento JM**, Heywood G, Rubin J, Ilstrup DM, Nagorney DM, Que FG. Surgical treatment of neuroendocrine metastases to the liver: a plea for resection to increase survival. *J Am Coll Surg* 2003; **197**: 29-37 [PMID: 12831921 DOI: 10.1016/S1072-7515(03)00230-8]

4   **Norlén O**, Stålberg P, Zedenius J, Hellman P. Outcome after resection and radiofrequency ablation of liver metastases from small intestinal neuroendocrine tumours. *Br J Surg* 2013; **100**: 1505-1514 [PMID: 24037573 DOI: 10.1002/bjs.9262]

5   **Daskalakis K**, Karakatsanis A, Stålberg P, Norlén O, Hellman P. Clinical signs of fibrosis in small intestinal neuroendocrine tumours. *Br J Surg* 2017; **104**: 69-75 [PMID: 27861745 DOI: 10.1002/bjs.10333]

6   **Chen L**, Song Y, Zhang Y, Chen M, Chen J. Exploration of the Exact Prognostic Significance of Lymphatic Metastasis in Jejunoileal Neuroendocrine Tumors. *Ann Surg Oncol* 2018; **25**: 2067-2074 [PMID: 29748891 DOI: 10.1245/s10434-018-6511-9]

7   **Isik A**, Firat D, Yilmaz I, Peker K, Idiz O, Yilmaz B, Demiryilmaz I, Celebi F. A survey of current approaches to thyroid nodules and thyroid operations. *Int J Surg* 2018; **54**: 100-104 [PMID: 29709542 DOI: 10.1016/j.ijsu.2018.04.037]

8   **Capurso G**, Rinzivillo M, Bettini R, Boninsegna L, Delle Fave G, Falconi M. Systematic review of resection of primary midgut carcinoid tumour in patients with unresectable liver metastases. *Br J Surg* 2012; **99**: 1480-1486 [PMID: 22972490 DOI: 10.1002/bjs.8842]

9   **Niederle B**, Pape UF, Costa F, Gross D, Kelestimur F, Knigge U, Öberg K, Pavel M, Perren A, Toumpanakis C, O'Connor J, O'Toole D, Krenning E, Reed N, Kianmanesh R; Vienna Consensus Conference participants. ENETS Consensus Guidelines Update for Neuroendocrine Neoplasms of the Jejunum and Ileum. *Neuroendocrinology* 2016; **103**: 125-138 [PMID: 26758972 DOI: 10.1159/000443170]

10 **National Comprehensive Cancer Network**. NCCN guidelines version 3.2017. Neuroendocrine tumors of the gastrointestinal tract, lung and thymus (carcinoid tumors). Available from: URL: https://www.nccn.org/professionals/physician_gls/pdf/neuroendocrine.pdf

11 **Daskalakis K**, Karakatsanis A, Hessman O, Stuart HC, Welin S, Tiensuu Janson E, Öberg K, Hellman P, Norlén O, Stålberg P. Association of a Prophylactic Surgical Approach to Stage IV Small Intestinal Neuroendocrine Tumors With Survival. *JAMA Oncol* 2018; **4**: 183-189 [PMID: 29049611 DOI: 10.1001/jamaoncol.2017.3326]

12 **Blažević A**, Zandee WT, Franssen GJH, Hofland J, van Velthuysen MF, Hofland LJ, Feelders RA, de Herder WW. Mesenteric fibrosis and palliative surgery in small intestinal neuroendocrine tumours. *Endocr Relat Cancer* 2018; **25**: 245-254 [PMID: 29255095 DOI: 10.1530/ERC-17-0282]

13 **Wu L**, Fu J, Wan L, Pan J, Lai S, Zhong J, Chung DC, Wang L. Survival outcomes and surgical intervention of small intestinal neuroendocrine tumors: a population based retrospective study. *Oncotarget* 2017; **8**: 4935-4947 [PMID: 27903960 DOI: 10.18632/oncotarget.13632]

REVIEW

Helicobacter pylori and extragastric diseases: A review

Antonietta Gerarda Gravina, Rocco Maurizio Zagari, Cristiana De Musis, Lorenzo Romano, Carmelina Loguercio, Marco Romano

Antonietta Gerarda Gravina, Cristiana De Musis, Lorenzo Romano, Carmelina Loguercio, Marco Romano, Dipartimento di "Medicina di Precisione", UOC Epatogastroenterologia, Università della Campania "Luigi Vanvitelli", Napoli 80131, Italy

Rocco Maurizio Zagari, Dipertimento Di Scienze Mediche e Chirurgiche, Università di Bologna, Bologna 40138, Italy

ORCID number: Antonietta Gerarda Gravina (0000-0001-8049 -0115); Rocco Maurizio Zagari (0000-0001-9949-8619); Cristiana De Musis (0000-0001-7011-5047); Lorenzo Romano (0000-0002-6581-7930); Carmelina Loguercio (0000-0002-6863 -6510); Marco Romano (0000-0002-3271-349X).

Abstract

Helicobacter pylori (*H. pylori*) infection is very common and affects approximately half of the world population. It causes gastric diseases, but some authors have reported an association of *H. pylori* infection with other systemic manifestations beginning in 1994. The list of potential effects of *H. pylori* outside the stomach includes a number of extragastric manifestations and we focused on neurological, dermatological, hematologic, ocular, cardiovascular, metabolic, allergic, and hepatobiliary diseases. This review discusses these important reported manifestations that are not related to the gastrointestinal tract.

Key words: *Helicobacter pylori*; Extragastric disease; Neurological diseases; Dermatological diseases; Hematologic diseases; Ocular diseases; Cardiovascular diseases; Metabolic diseases; Allergic diseases; Hepatobiliary diseases

Core tip: *Helicobacter pylori* (*H. pylori*) infection is a common infection that can cause gastric and extragastric diseases. A considerable amount of evidence links *H. pylori* infection with extragastric diseases, and in many of these diseases there is a clear beneficial effect of eradication therapy. This review summarizes the *H. pylori*-related extragastric manifestations of major interest that have been reported in the scientific literature, such as neurological, dermatological, hematologic, ocular, cardiovascular, metabolic, allergic

and hepatobiliary disease manifestations.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative, microaerophilic, spiral-shaped and flagellated bacterium infecting about half the world's population whose main reservoir is the human stomach. The prevalence of infection varies by geographic area, age, ethnicity and socioeconomic status; in fact, the prevalence is higher in developing countries and in those with poor socio-economic conditions[1-4]. *H. pylori* possesses microbiological characteristics that allow it to survive in extremely adverse conditions such as the gastric acidic environment. Transmission of the infection occurs mainly through the oral-fecal route[5], in particular through contaminated water and food. Oral-oral transmission is also possible, as shown by the isolation of the bacterium in saliva and dental plaque[6].

H. pylori infection is the main cause of chronic gastritis and peptic ulcer disease[7]. *H. pylori* has a determinant pathogenic role in the development of distal gastric adenocarcinoma and gastric mucosa associated lymphoid tissue (MALT) lymphoma; in fact, it can contribute to gastric carcinogenesis by stimulating gastric cell proliferation without counterbalancing with adequate apoptosis[8,9]. The spectrum of gastroduodenal diseases associated with the evolution of *H. pylori* infection is wide. A large proportion of infected subjects (approximately 80%-85%) develop mild antrum and body gastritis associated with an alteration of gastric homeostasis characterized by hypergastrinemia with normal levels of gastric acid secretion. These subjects are unlikely to develop severe clinical conditions. Approximately 10%-15% of infected individuals develop prevalent antrum gastritis, in which hypergastrinemia is associated with increased gastric secretion with the possible development of duodenal ulceration[10]. A lower percentage (approximately 1%-2%) develop a prevalent body gastritis in response to the infection that is associated with multifocal atrophic gastritis, increased gastrinemia, hypo-chlorhydria and the possible development of gastric adenocarcinoma. The reason why *H. pylori* infection is associated with different gastric phenotypes with different clinical outcomes is not fully known, but it is likely that the interaction among bacterial virulence factors (*e.g.*, CagA, urease, VacA, and babA2), environmental factors (*e.g.*, socioeconomic conditions, nutrition, and exposure to toxic substances) and genetic

substrates of the host [*e.g.*, IL1B gene cluster and tumor necrosis factor-α (*TNF*α) gene polymorphism] play an important role[11]. The first reports of the association between *H. pylori* infection and extra-gastric diseases were by Mendall *et al*[12] in 1994. There are several clinical extra-gastric manifestations associated with *H. pylori* infection that have been reported to date, making this a very interesting and debated topic. We summarize the data in the literature about extra-digestive diseases associated with *H. pylori* infection and focus our attention on the following conditions that are potentially linked to *H. pylori* infection: neurological, dermatological, hematologic, ocular, cardiovascular, metabolic and allergic diseases. For some of these diseases, only one association is described without a clear explanation of the pathogenic mechanism; however, for others, as we will see later, the association is so strong and the pathogenic mechanism is so clear that guidelines for the treatment of *H. pylori* infection suggest that in these conditions, *H. pylori* infection should be determined and, if present, should be treated with eradication therapy[13].

NEUROLOGICAL DISEASES

Several neurological disorders are associated with *H. pylori* infection. There are studies in the literature that report a positive predictive value between *H. pylori* infection and stroke; however, in 2013, in a prospective cohort analysis performed on 9885 subjects with stroke, Chen *et al*[14] not only reported no increased mortality but actually found that people infected with *H. pylori* had a lower mortality than the general population. In a recent metanalysis, Wang *et al*[15] demonstrated that chronic *H. pylori* infection and the presence of CagA-positive strains were statistically significant risk factors for ischemic stroke. The underlying pathogenic mechanism is not yet known, but it has been hypothesized that *H. pylori* increases the expression of a number of mediators of inflammation and activates platelets and factors involved in coagulation[16]. Difference in the study population and in the methods to assess *H. pylori* infection might partially explain the discrepancy between different studies.

Another neurologic disease that has been linked to *H. pylori* infection is Alzheimer's disease (AD). There are several studies concerning *H. pylori* and dementia. Huang *et al*[17] showed a 1.6 times greater risk of developing AD in *H. pylori*-infected people than in non-infected people, supporting a possible role of *H. pylori* in the pathophysiology of AD. Roubaud Baudron *et al*[18] also reported a 1.5 times greater risk of developing dementia over a 20-year follow-up period in infected people than in non-infected people. Beydoun *et al*[19] also reported an association between *H. pylori* infection and reduced cognitive ability. There are also several studies that report that eradication of infection can positively influence the manifestations of AD[20-22]. In 2016, Kountouras *et al*[23] showed that in patients

with AD and *H. pylori* infection there was an increased prevalence of apolipoprotein E (ApoE) 4 polymorphism compared with non-infected patients. The ApoE 4 polymorphism is the strongest genetic risk factor for AD[23,24]. In 2009, the same author reported significantly higher levels of anti-*H. pylori*-specific antibodies (anti-*H. pylori* IgG) in the cerebrospinal fluid (CSF) and serum from patients with AD than that in the CSF and serum from age-matched subjects with normal cognition. The same research group demonstrated a significant correlation between the severity of disease and levels of anti-*H. pylori* IgG in the CSF of these patients[25]. One hypothesis to explain the association between *H. pylori* and AD is that *H. pylori* might access the brain *via* an oral-nasal-olfactory pathway thus leading to neurodegeneration. In fact, olfactory performance is reduced in 90% of patients with AD, and significant olfactory bulb atrophy, as evaluated by imaging techniques, is present in these patients[26-29]. Another hypothesis is that *H. pylori* may access the brain *via* monocytes infected with *H. pylori* (due to *H. pylori* replication in autophagic vesicles) through a disrupted blood-brain barrier (BBB), which is also called the "Trojan horse theory"; this may lead to increased production of inflammatory mediators, such as TNF-α, which in turn may cause BBB disruption by up-regulation of metalloproteinases[30]. A third hypothesis is that *H. pylori* may access to brain through a fast retrograde neural pathway from the gastrointestinal tract (GIT), leading to neurodegeneration[30]. A study conducted in Japan[31] did not confirm the association between *H. pylori* infection and AD. However the high prevalence of *H. pylori* in controls might partially explain the difference with studies conducted in Western countries where the prevalence of the infection is lower. Furthermore, Chang *et al*[32] in partial support of a role for *H. pylori* in AD, demonstrated that eradication of the infection led to a decreased progression of the neurological disease.

Multiple sclerosis (MS) is a chronic demyelinating disease that affects the central nervous system. Mohebi *et al*[33] showed an inverse relationship between *H. pylori* infection and MS; however, other authors have shown positivity of markers for optic neuromyelitis in *H. pylori*-positive patients. The administration of *H. pylori* SS1 antigen in an experimental model of MS [experimental autoimmune encephalomyelitis (EAE)], indicates the presence of immunomodulating properties of *H. pylori*, suggesting a possible effect of *H. pylori* infection in the pathophysiology of MS[34]. Cook *et al*[35] showed a probable protective role of *H. pylori* against EAE by inhibiting both Th1 and Th17 responses. *H. pylori* infection seems to be a risk factor for the development of antiaquaporin 4 (AQP4) antibodies in MS, through molecular mimicry between AQP4 and bacterial AQP[14]. Therefore, based on these studies, whether *H. pylori* infection may cause MS is still controversial.

Another disease of great neurological interest for which an association with *H. pylori* infection has been reported is Parkinson's disease (PD). PD is caused by the degeneration of the dopaminergic neurons of the substantia nigra pars compacta of the basal ganglia system. A meta-analysis by Shen *et al*[36] in 2017, evaluating eight eligible studies involving 33125 participants, demonstrated that *H. pylori* infection might be associated with the risk of PD. A recent study by Huang *et al*[37] in the general population in Taiwan demonstrated that *H. pylori* infection was significantly associated with an increased risk of PD among individuals who were ≥ 60 years old but not among those < 60 years old. *H. pylori* infection may affect L-3,4-dihydroxyphenylalanine (L-dopa) bioavailability, which is used in the treatment of PD, by disrupting the duodenal mucosa, which is the site of absorption of L-dopa[38]. The eradication of the infection appears to be associated with greater bioavailability of L-dopa and is associated with better clinical outcomes[39,40]. Several studies have demonstrated that pro-inflammatory cytokines associated with chronic gastrointestinal disease can induce brain inflammation through a disruption of the BBB and death of dopaminergic neurons and may eventually be responsible for parkinsonism[41-43]. The association between PD and *H. pylori* infection seems, therefore, to be strongly supported by literature reports.

Guillain-Barré syndrome (GBS) is an acute autoimmune neuropathy characterized by progressive paralysis of the limbs with a distal-proximal pattern (the legs being affected first and the arms later). It can cause life-threatening complications, particularly if there is respiratory muscle involvement or autonomic nervous system involvement. The disease is usually triggered by an infection. *Campylobacter jejuni* (*C. jejuni*), the most prevalent cause of bacterial gastroenteritis, has been associated with GBS and the possible pathogenic mechanism involves molecular mimicry between peripheral nerve gangliosides and *C. jejuni* lipopolysaccharides (LPSs). In fact sialic acid, a characteristic component of human gangliosides, is present among the surface antigens of *C. jejuni*. *H. pylori* has a high-molecular weight sequence in LPSs that is detected in one third of *C. jejuni* serotypes. Therefore a molecular mimicry between *H. pylori* antigens and peripheral nerve gangliosides might be responsible for the association between *H. pylori* infection and GBS[16,44,45]. Some authors demonstrated that the presence of serum anti-*H. pylori* IgG in patients with GBS was significantly higher than that in controls. They also demonstrated that the CSF was positive for anti-*H. pylori* IgG in 80% of patients and in only 20% of controls[16]. Chiba *et al*[46] demonstrated IgG antibodies to vacuolating cytotoxin A (VacA) of *H. pylori* in the CSF of patients with GBS. In this study, the authors found sequence homology between VacA and the human ATPase A subunit, suggesting that antibodies to VacA might bind to ion channels in Schwann cells, resulting in the demyelination of motor neurons in these patients. A limitation of all these studies is the small sample size and, for this reason, studies with a greater sample size are needed to demonstrate a real cause-

effect relationship between *H. pylori* and GBS.

DERMATOLOGICAL DISEASES

Rosacea is the most common dermatological disease associated with *H. pylori* infection. It is a chronic facial dermatitis that manifests as erythema and cutaneous lesions characterized by much dilated red superficial capillaries, called telangiectasia, and its etiology remains unknown. Although the etiopathogenesis is not fully known, an element commonly found in patients with rosacea is the presence of gastrointestinal disorders. Several studies have suggested a potential relationship to *H. pylori* infection; however, the correlation between *H. pylori* infection and rosacea is still debated. Our group has demonstrated *H. pylori* infection in 48.9% of patients with rosacea, while only 26.7% of patients in the control group were infected with *H. pylori*. We demonstrated an improvement with partial or total regression of rosacea skin lesions after successful *H. pylori* eradication in 96.9% of patients[47]. Argenziano *et al*[48] reported *H. pylori* infection in 81% of patients with rosacea, and almost all of the patients housed CagA-positive strains. El-Khalawany *et al*[49] demonstrated that papular rosacea responded better to eradication therapy than erythematous rosacea did. Based on the majority of studies one may therefore suggest to test for and eradicate *H. pylori* infection in patients with rosacea.

Psoriasis is an autoimmune chronic inflammatory disease of the skin that is not infectious or contagious and is usually of a chronic and recurring nature. The association between *H. pylori* infection and psoriasis is still controversial. In fact some authors demonstrated neither a significant relationship between psoriasis and *H. pylori*[50,51], nor a significant relationship between psoriasis severity and the serum levels of IgG anti-*H pylori*[51] . However, other authors demonstrated that *H. pylori* infection is more common in patients with psoriasis than in healthy controls[52,53] and that prevalence of *H. pylori* infection was higher in patients with severe psoriasis (79%) compared to those with moderate (69.5%) or mild (46.2%) disease[53,54]. Finally, in support of a causative role for *H. pylori* in psoriasis, an interventional study by Onsun *et al*[55] demonstrated that eradication of *H. pylori* infection caused more rapid improvement than treatment with acitretin alone.

Chronic urticaria (CU) is characterized by the appearance of a more or less itchy rash, and its unique lesion is the wheal. Some research groups have reported a higher prevalence of *H. pylori* infection in patients with CU[56,57]. Campanati *et al*[58] demonstrated no difference in the presence of *H. pylori* infection between patients with CU and apparently healthy people, but they reported a significant improvement in skin lesions after eradication therapy. Yoshimasu *et al*[59] demonstrated that patients with CU infected with *H. pylori* had a cure rate of approximately 56% and that

none of the patients with *H. pylori* infection were cured if they were treated with antihistamine therapy alone.

Data regarding the association between alopecia aerata (AA) and *H. pylori* infection are discordant. In fact, some authors have reported a higher prevalence of infection in patients with AA, but other authors did not confirm this association[60,61]. Differences in methodology might account for the discrepancy of results related to the role of *H. pylori* in CU or AA and, therefore, larger population studies and controlled trials to obtain more accurate evidence about these associations are needed.

Autoimmune bullous diseases (AIBD) are a group of dermatological diseases including pemphigus, pemphigoid, epidermolysis bullosa acquisita, dermatitis herpetiformis, and linear immunoglobulin A disease[62,63]. Very few published studies have investigated the association between AIBD and *H. pylori* infection. Sagi *et al*[64] and Mortazavi *et al*[65] demonstrated that anti-*H. pylori* IgG was higher in patients with AIBD than in controls. In the Mortazavi *et al*[65] study, the prevalence of *H. pylori* infection was as high as 79.3% in the AIBD group.

Schöenlein-Henoch purpura (SHP) is an immune condition characterized by the deposition of immunoglobulin A in the skin and in other organs, such as the kidneys, joints, and gastrointestinal tract. The onset of this disease is characterized by the appearance of purple skin lesions. There are a few reports that support the association between *H. pylori* infection and SHP, demonstrating an improvement in skin lesions following the successful eradication of the infection[66-68]. Clinical studies with a great sample size are necessary prior to draw a firm conclusion regarding the role of *H. pylori* in SHP.

HEMATOLOGIC DISEASES

Iron deficiency anemia (IDA) is well known to be associated with *H. pylori* infection. In 1991, Blecker *et al*[69] described a case of hemorrhagic gastritis linked to *H. pylori* infection and showed a possible relationship between *H. pylori* infection and IDA. Subsequently, 5 meta-analyses concluded that there was a close association between infection and IDA[70-74]. Guidelines for the treatment of *H. pylori* infection indicate that the infection should be eradicated in cases of IDA[13]. The reason why IDA is not always associated with *H. pylori* infection is not known. Azab *et al*[75] studied the role of hepcidin, which is a small protein responsible for regulating iron recycling and the balance of iron in the body. Hepcidin, which is produced in the liver, regulates the absorption of iron from enterocytes and its release from macrophages[76]. *H. pylori* infection upregulates hepcidin serum levels, thus attenuating the response to iron therapy[77]. Senkovich *et al*[78] demonstrated that *H. pylori* is able to acquire iron from host transferrin and lactoferrin. Yokota *et al*[79] demonstrated that some polymorphisms within the *H. pylori* neutrophil-

activating protein were more frequent in strains from IDA patients than in those from non-IDA patients. *H. pylori* that harbors these polymorphisms internalizes iron more rapidly than other strains[79]. Inorganic iron dissolves best in a highly acidic environment, and *H. pylori* infection may reduce the bioavailability of dietary iron. Patients infected with CagA-positive strains and those with refractory IDA have high serum levels of TNFα, which is a pro-inflammatory cytokine that can induce anemia[80,81]. Hershko *et al*[82] demonstrated that 64%-75% of patients with IDA and *H. pylori* infections had a complete disappearance of IDA after *H. pylori* eradication. Other possible causes of IDA in patients with *H. pylori* infections are chronic or acute bleeding due to erosive gastritis or concomitant therapy with non-steroidal anti-inflammatory drugs including aspirin[83-86].

Vitamin B12 is the coenzyme of many important enzymatic reactions in the human body that lead to DNA synthesis[87]. Lahner *et al*[88] showed an association between low serum levels of vitamin B12 and *H. pylori* infection in a systematic review evaluating 17 studies involving 2454 patients. Homocysteine is a component of the vitamin B12 metabolic pathway[89], and some authors have demonstrated a relationship between low serum levels of vitamin B12 and increases in serum homocysteine associated with *H. pylori* infection[90]. Increased serum levels of vitamin B12 and decreased serum levels of homocysteine have been reported after *H. pylori* eradication[87].The pathophysiological mechanism of the association between vitamin B12 deficiency and *H. pylori* infection is also unknown. The absorption of vitamin B12 may be compromised in corpus-predominant *H. pylori*-related gastritis. Chronic vitamin B12 deficiency can cause a pernicious anemia and peripheral neuropathy and lesions of the spinal cord[91].

Primary immune thrombocytopenia (ITP), which was previously called idiopathic thrombocytopenic purpura and autoimmune thrombocytopenic purpura, is an autoimmune disorder characterized by an isolated thrombocytopenia (a peripheral blood platelet count of 100×10^9/L) in the absence of other causes[87]. The first case of association between *H. pylori* infection and ITP was described in Spain in 1999[92]. In the world literature, there are many authors who have reported this association, and a significant increase in platelet count following the eradication of *H. pylori* infection varies from 32% to 100% in Italian studies[93,94] and from 26% to 100% in the overall literature[95,96]. There are only a few published studies that do not describe a positive association between *H. pylori* infection and ITP, but this may be explained by the low prevalence of the infection in these countries[97-100]. *H. pylori*-infected patients who have a particular polymorphism in IL-β, the IL-β (-511) T allele, are more likely to develop ITP than uninfected ITP patients[80,101]. The pathogenesis of ITP associated with *H. pylori* infection is most likely multifactorial. Various mechanisms involved in this autoimmune process in patients with *H. pylori* infection

have been described, and one mechanism does not exclude the other. One of these mechanisms is the modulation of the Fcγ receptor balance, which is related to the activation of monocytes/macrophages and the relationship to the inhibitory receptor FcγRIIB. Monocytes from *H. pylori*-infected patients exhibited an enhanced phagocytic capacity and low levels of inhibitory FcγRIIB, leading to increased monocyte function with autoreactivity with B and T lymphocytes. This may cause the production of autoantibodies by lymphocyte B against circulating platelets. Eradication of *H. pylori* in ITP causes an upregulation of FcγRIIB expression in monocytes, increased platelet recovery and suppression of antigen presentation by macrophages with the subsequent inhibition of T- and B-cell responses to platelet antigens[87,102]. Another mechanism potentially involved in *H. pylori*-associated ITP is molecular mimicry between platelet surface glycoproteins, including glycoprotein IIIa, and amino acid sequences of virulence factors, such as VacA, CagA and urease B[87,103]. Anti-*H. pylori* IgG can induce opsonization of platelets by binding to *H. pylori*, von Willebrand's factor, and GPIb[102,104].

The putative mechanisms responsible for the association between *H. pylori* infection, IDA, Vitamin B12 deficiency and ITP are summarized in Figure 1.

Other unrecognized hematologic disorders that are associated with *H. pylori* infection are autoimmune neutropenia (AN), anti-phospholipid syndrome (AS), and plasma cell dyscrasias (PCD). The association between AN and *H. pylori* infection was described for the first time by Cicconi *et al*[105] in 2001 and was subsequently described by other authors[105,106]. AN (defined as 400 neutrophils per μL) may dramatically improve after *H. pylori* eradication[87,107,108]. The association between *H. pylori* infection and PCD, including monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma, solitary plasmacytomas, plasma cell leukemia, Waldenstrom macroglobulinemia and other chronic myeloproliferative diseases of B lymphocytes, is supported by some authors but not by others[107,109-111]. This relationship maybe a result of chronic antigenic stimulation of B lymphocytes by *H. pylori*[84].

OCULAR DISEASES

Ocular diseases described in association with *H. pylori* infection are open-angle glaucoma (OAG), central serous chorioretinitis (CSC) and blepharitis (B). There are studies that show a prevalence of *H. pylori* infection that is approximately two-fold higher in patients with OAG than in controls. However, other authors including Galloway *et al*[112] and Kurtz *et al*[113] did not find a higher prevalence of infection in patients with open-angle glaucoma. Zeng *et al*[114] published a meta-analysis about this association, evaluating ten studies, and suggested a statistically significant association between *H. pylori* infection and OAG. In further support of an association between *H. pylori* infection and
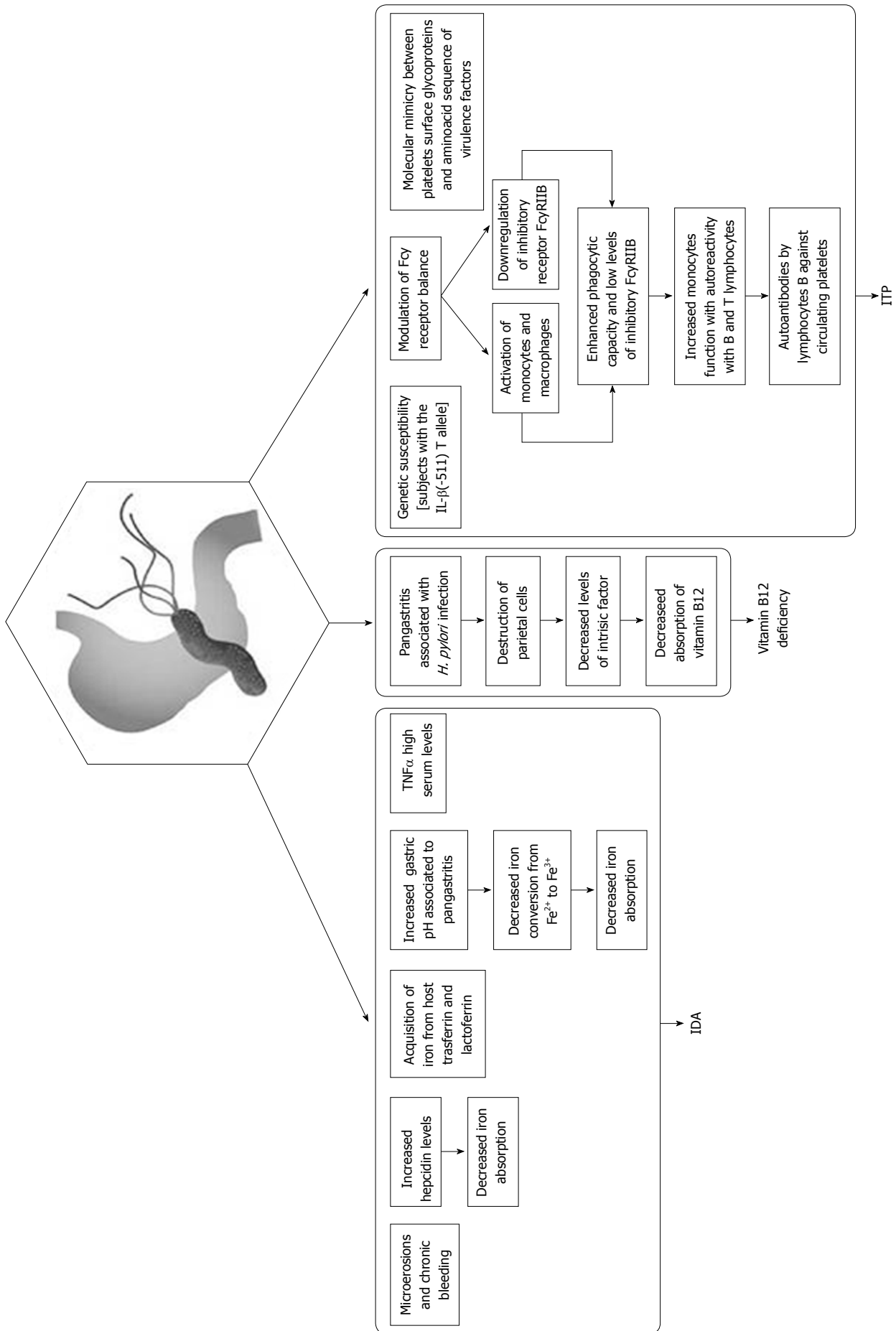
Figure 1 Hematologic diseases associated to *Helicobacter pylori* infection.

OAG, successful eradication of the infection has been reported to lead to an improvement in ocular pressure parameters in treated patients compared with the patients who do not have the infection eradicated[80].

CSC is an ocular disease that causes a temporary reduction in central vision and usually affects only one eye. In the different phases of activity, it is characterized by the presence of liquid passing under the retina, which tends to accumulate under the macula. This results in blurred or distorted vision, with a reduction in visual acuity that can persist even after reabsorption of the fluid if it does not take place rapidly[115]. In a systematic review and meta-analysis about the risk factors for CSC published in 2016, Liu *et al*[116] concluded that *H. pylori* infection was a possible risk factor for the occurrence of CSC. Our research group also evaluated this association in a retrospective observational case series and demonstrated that the prevalence of *H. pylori* infection was 78.2% in CSC patients and 43.5% in control subjects[117]. *H. pylori* eradication may lead to an improvement in CSC[115,118,119]. In particular, Zavoloka *et al*[120] demonstrated that *H. pylori* eradication caused a decrease in the disease duration of 3 mo and in the recurrence frequency of 45.6 % and led to an improved long term prognosis. In fact, after two years, the visual acuity increased, the scotoma frequency decreased and the metamorphopsia frequency decreased.

Blepharitis is characterized by non-granulomatous inflammation of the eyelid margin. It remains difficult to ascertain whether its association with *H. pylori* infection is real because the data are highly discordant[121,122].

CARDIOVASCULAR DISEASES

The association of *H. pylori* infection with cardiovascular diseases (CD) represents one of the fields of study that has attracted the most attention in the scientific community, despite the evident difficulties in identifying the specific role of *H. pylori* in the pathogenic processes of CD, including coronary atherosclerotic disease (CAD), stroke and myocardial infarction. Mendall *et al*[12] were the first to describe a significant association between *H. pylori* infection and the development of CAD in male subjects aged 45-65 years in a prospective study. CAD is due to dysfunction of the vessel endothelium associated with a simultaneous remodeling of the vessel wall, which is accompanied by an increase in blood pressure, a local inflammatory state and blood clotting; these are all phenomena that overall converge towards the formation of atherosclerotic plaques that are frequently unstable and susceptible to breakage. A possible rupture may compromise the blood circulation and lead to a myocardial infarction (MI). There are multiple causal factors that are involved in the pathogenesis during the initial stages of disease progression, including smoking, arterial hypertension, the presence of mixed dyslipidemia (alterations in total cholesterol levels, low density lipoprotein (LDL) and triglycerides) associated with a simultaneous reduction in high density lipoprotein

(HDL) cholesterol, obesity, diabetes mellitus, and the presence of hyperhomocysteinemia and increased coagulation factors[123,124]. The hypothesis that infectious factors may play a role in the pathogenesis of CAD has developed only recently, probably stimulated by evidence that, despite the predisposing factor pattern, there is still a part of the population affected by CAD in which the origin and progression of the disease is inexplicable. Indeed, Lai *et al*[125] have shown that *H. pylori* infection increases the risk of acute coronary artery disease, even in the absence (or after the removal) of risk factors. The most likely pathogenic mechanism of the relationship between *H. pylori* and CAD is the initiation of a chronic inflammatory process associated with infection; however, it should be clarified if the role of *H. pylori* is to trigger the disease or only to accelerate its clinical course. The particular role of *H. pylori* in the pathogenesis of CAD is thought to be related to its ability to trigger a persistent chronic inflammatory state that is established within the gastric epithelium but that can also cause systemic inflammatory effects[126]. In the stomach, VacA and urease contribute to the destruction of tight junctions, and this may allow the bacterial agents that breach the lamina propria to come into contact with the cells of the immune system[127]. *H. pylori* antigens can either interact directly with the vascular endothelium or may form complexes with LDL/oxLDL cholesterol[128]. The subacute inflammation present in chronic diseases, such as CAD, can be determined by the activation of the cells of the immune system or epithelial cells, through pattern recognition receptors (PRRs) that specifically recognize pathogen-associated molecular pattern[129]. Both endothelial cells and macrophages present in atherosclerotic lesions show an overexpression of PRRs, such as TLR4, CD14 and TLR2, that are specialized for recognizing bacterial LPS[130,131]. Xu *et al*[131] suggested a possible pathophysiological link between lipids, *H. pylori* infection, inflammatory status and CAD based on the overexpression of TLR4 on the surface of macrophages induced by pro-oxidant oxidized LDL. The inflammatory hypothesis was confirmed by several studies conducted with the intent of identifying an association between the increase in inflammatory markers and CAD. Li *et al*[132] and Libby *et al*[133] have highlighted a relationship between CAD and some of the most important inflammation markers, such as C-reactive protein (CRP), interleukin-6 (IL-6) and TNF-α, which are all expressed at higher levels in patients with atherosclerosis than in controls. CRP is an important acute phase protein that is associated with infectious and inflammatory processes or tissue damage; therefore, it is a reliable marker of endothelial dysfunction in CAD[134,135]. A second hypothesis about the relationship between *H. pylori* infection and the pathogenesis of CAD is that bacterial antigens can induce T- and B-cell expansion and cause self-reactive antibody production by molecular mimicry. As an example of molecular mimicry, Matsuura *et al*[136] proposed that heat shock protein 60 derived from *H.*

pylori (Hp-HSP60) may potentially be related to the pathogenesis of CAD through the stimulation of Th1 lymphocytes, which are induced to produce INF-γ and IL-12, or through the activation of macrophages important in forming atherosclerotic plaques. An analysis of the literature clearly shows that serum positivity for CagA is closely associated with heart disease, including atherosclerosis, unstable angina, cardiac syndrome X and coronary artery disease[137-139]. CagA is the most important virulence factor of *H. pylori*. CagA-positive strains are associated with increased IL-8 production, which is a marker of inflammation[140]. The atherosclerotic cascade is notoriously characterized by an increase in the expression levels of inflammation markers, such as CRP and some interleukins. Discordant data about the association between *H. pylori* infection and CAD are present in the literature. In fact, Manolakis *et al*[141] did not find a correlation between CRP levels and *H. pylori* infection. Similarly, Carter *et al*[142] did not find any correlation between *H. pylori* infection and fibrinogen or von Willebrand's factor levels.

Discordant data about the relationship between *H. pylori* and myocardial infarction (MI) also exist. In 2013, Ikeda *et al*[143] demonstrated that only CagA-positive *H. pylori* strains were associated with an increased risk of MI. In a meta-analysis of 26000 patients, Liu *et al*[144] demonstrated the existence of a significant association between *H. pylori* infection and the risk of MI, whereas Hughes *et al*[145] showed a parallel decline of MI and duodenal ulcers in people born from 1930 to 1980. This study showed that the decline in MI was temporally related to a decline in duodenal ulcers and, by inference, *H. pylori* infection. The discordant data make it impossible to provide an unambiguous definition of *H. pylori* as a causal factor for cardiovascular diseases.

METABOLIC DISEASES

H. pylori infection has also been identified as related to alterations in glycolipid metabolism. The role of *H. pylori* infection in diabetes mellitus (DM), insulin resistance syndrome (IR), and metabolic syndrome (SMet) is still rather controversial, and in some cases, appears to bear marginal weight.

The association between *H. pylori* infection and DM has been suggested recently but still appears rather unclear[146]. In a study of a Chinese population, Hsieh *et al*[147] demonstrated how high levels of glycated hemoglobin (HbA1c) were significantly associated with *H. pylori* infection in patients aged > 65 years. Bégué *et al*[148] demonstrated that eradication of *H. pylori* infection in patients with type 1 DM might be associated with better control of glycemia by evaluating HbA1c, which is an important indicator of long-term glycemic control, within two years after the eradication of the infection. In contrast, in 141 patients with type 2 DM, Demir *et al*[149] demonstrated no significant differences in either blood glucose levels or HbA1c values in patients

with *H. pylori* infections compared with controls. In a cross-sectional study including 1285 subjects aged 19-85 years, Yang *et al*[150] suggested the existence of a correlation between *H. pylori* infection and DM. According to Horikawa *et al*[151], the glycemic control of patients with DM was worse in the presence of *H. pylori* infections, in particular in infections with CagA-positive strains. A few studies demonstrated that eradication could lead to a benefit for glycemic control[152,153]. This has not been confirmed by other studies[154]. A meta-analysis by Wang *et al*[155] demonstrated the presence of higher levels of inflammatory markers in diabetic patients than in controls. Jeon *et al*[156] demonstrated that IL-6 levels and CRP levels, when used as inflammatory markers, appeared to be very similar in patients with DM, both in patients who were *H. pylori* positive and in those who were negative. As it is now known, chronic *H. pylori* infection is responsible for a low-level inflammatory state of the gastric mucosa, which is defined as the biological response of the mucosa to pathogens, that can involve different regions depending on the host phenotype. Chronic gastritis, such as that found in type 2 DM, atherosclerosis and SMet, is associated with an increase in circulating pro-inflammatory cytokine levels that are capable of interfering with both local and systemic metabolic processes[157]. Studies have also been conducted evaluating complications associated with DM. Wang *et al*[158] described a possible correlation between *H. pylori* infection and the risk of nephropathy or neuropathy in an Asian population; however, some other authors did not confirm this correlation[159]. Vafaeimanesh *et al*[160] have demonstrated the existence of an association between diabetic patients with microalbuminuria and *H. pylori* infection. Microalbuminuria, as well as neuropathy and heart disease, are very common complications in patients with DM, and patients with CagA-positive *H. pylori* infection are at a greater risk of developing these complications[152,161].

Vafaeimanesh *et al*[160] also focused on the possible correlation between *H. pylori* infection and the development of IR. IR and *H. pylori* infection share pathogenic mediators that are potentially involved in the pathophysiology of the SMet[161]. Zhou *et al*[162] showed that *H. pylori* may induce hepatic IR by interfering with the c-Jun/miR-203/SOCS3 pathway, both in humans and in animal models. An analysis of the literature shows that *H. pylori* eradication can improve IR, although this resolution seems to be associated with a progressive increase in BMI and cholesterol levels, as *H. pylori* suppresses ghrelin, which is the hormone responsible for increased appetite, which may explain the weight gain associated with the eradication of infection[154]. However, one can hypothesize that the resolution of the symptoms related to infection may lead to recovery of a normal weight. HOMA-IR, which is a model for measuring insulin homeostasis, is higher in patients with *H. pylori* infection than in control[163-165]. However, this finding has not been confirmed by other

studies[166].

Considering the aforementioned role of *H. pylori* in IR, there have been several studies in the literature that demonstrate a higher prevalence of SMet in patients with *H. pylori* infection[167]. Some studies have revealed changes in the lipid profiles of patients associated with the low-level inflammatory status induced by *H. pylori* infection; this has been demonstrated by Niemelä *et al*[168] in a study involving a Finnish population, in which serum cholesterol and triglyceride levels were higher in infected male subjects. Several studies have demonstrated the presence of these serum lipid profile alterations that can promote atherogenic processes[169].

ALLERGIC DISEASES

In the literature, a number of studies and meta-analyses have reported an inverse association between *H. pylori* infection and asthma, worldwide[170-172]. Blaser *et al*[173] showed the existence of an inverse relationship between *H. pylori* infection and the development of asthma or other allergic diseases, particularly in children and young people with an early onset of allergies. This relationship is controversial in adults, however, and has been confirmed mainly in cases in which the *H. pylori* strains are CagA-positive. Amberbir *et al*[174] showed that the relationship between *H. pylori* and rhinitis is not always inverse. *H. pylori* can almost completely protect against airway hyper-reactivity, broncho-alveolar eosinophilia, and lung inflammation, but this protection, which is strongly dependent on regulatory T (Treg) lymphocytes, is impaired by the complete eradication of the infection through antibiotic therapy[170]. The functionality of T reg cells depends on IL-18 production by dendritic cells (DCs) following exposure to *H. pylori*, in the absence of which, a neonatal tolerance to infection is not established. Treg cells are derived from cells with an IL-18 -/- or an IL-18R -/- phenotype that have no protective capabilities against asthma; moreover, IL-18 is fundamental for the conversion of CD4+ T-cells into CD25+ Foxp3+ Treg cells[175]. VacA toxin is the most important factor involved in protection against allergies. It appears to disrupt the T helper cell response to Treg cells. It stimulates Treg cells, but it also has the ability to interfere with antigen presentation and T-cell inhibition[176]. *H. pylori* strains expressing the active form of VacA are usually also CagA positive[177]. Most likely, CagA-positive strains elicit a greater response through modulation of inflammation by Treg cells, but it seems plausible that there is also a pronounced effect on the migration of Treg cells[178]. To validate this hypothesis, Engler *et al*[179] demonstrated that the administration of VacA to mouse models was able to provide allergy protection that was comparable to that of active infection. However, these effects seem to be more pronounced if this administration is carried out during the neonatal stage of life, probably because this represents a crucial period during which antigenic

tolerance develops, both in mice and in humans. *H. pylori* influences the immune system by shifting the balance of cytokines to the Th1 type, which suppresses allergic diseases that are dependent on the Th2 cytotype[180,181], protecting infected individuals from developing atopic disease. This might also explain the low prevalence of eosinophilic esophagitis in *H. pylori*-infected subjects[182]. When analyzing the association between *H. pylori* infection and allergies one should take into account the hygiene hypothesis and the reduced prevalence of allergic diseases in pet owners. In fact, *H. pylori* infection is associated with poor hygiene, crowding and low socio-economic status. Poor hygiene conditions and low socioeconomic status together with pet ownership may expose to other bacteria or antigens which may reduce the risk of allergic diseases. Therefore, in the interpretation of epidemiologic studies between H. pylori infection and allergies, possible confounding factors should be considered before drawing conclusions on putative cause-effect relationship[180-182].

HEPATOBILIARY DISEASES

Recent studies have shown the involvement of *H. pylori* in the etiopathogenesis of a number of liver diseases[183,184]. Polyzos *et al*[185] showed a higher serum level of anti-*H. pylori* IgG in patients with nonalcoholic fatty liver disease (NAFLD) than in non-NAFLD patients. Many other studies have shown an association between NAFLD and *H. pylori*[186,187]. *H. pylori* could be related to a worsening of the inflammatory status of the liver, regardless of the etiology of the underlying liver disease[184]. Fukuda *et al*[188] have proposed that because of the increase in gastric and intestinal mucosal permeability, *H. pylori* antigens could have access to the blood stream and reach the liver through the portal vein, thus causing liver damage. Some authors, such as Sumida *et al*[189], suggest that the eradication of *H. pylori* may play an important role in the treatment of nonalcoholic steatohepatitis (NASH), through the decrease of TNFα, one of the pro-inflammatory cytokines that, together with IL-1β, IL-6 and IL-8, is also directly correlated with the etiopathogenesis of IR, and of NASH[190,191]. Adiponectin is also involved in the etiopathogenesis of NAFLD. In fact adiponectin deficiency is associated with a pro-inflammatory condition, as it is observed in obesity and other metabolic disorders[192]. According to Polyzos *et al*[185] a higher prevalence of low levels of circulating adiponectin, as well as of higher levels of anti-*H. pylori* IgG and elevated TNFα are observed in NAFLD patients compared to controls.

The correlation between *H. pylori* and hepatic fibrosis was analyzed mostly in animal models. Ki *et al*[193] demonstrated in a murine model that *H. pylori* infection may accelerate hepatic fibrosis through increased TGF-β1-induced pro-inflammatory signaling pathways in hepatic stellate cells and that *H. pylori* infection might increase the risk of TGF-β 1-mediated tumorigenesis

Table 1 Literature reports in favor (pro) or against (con) of *Helicobacter pylori* involvement in a number of extragastric conditions

Neurologic diseases	Pro	Con
Stroke	Wang et al[15] Alvarez-Arellano et al[16]	Chen et al[14]
Alzheimer's disease	Huang et al[17] Roubaud Baudron et al[18] Beydoun et al[19] Kountouras et al[21] Kountouras et al[22] Kountouras et al[23] Santos et al[24] Kountouras et al[25] Zelaya et al[26] Attems J et al[27] Thomann et al[28] Förster et al[29] Chang et al[32]	Shiota et al[31]
Multiple sclerosis	Chen et al[14]	Mohebi et al[33] Cook et al[35]
Parkinson's disease	Shen X et al[36] Huang HK et al[37] Fasano et al[38] Tan et al[39] Mridula et al[40] Candelario-Jalil et al[41] Lo et al[42] Dobbs et al[43]	
Guillain-Barré syndrome	Kountouras et al[44] Moran et al[45] Chiba et al[46]	
Dermatological diseases	Pro	Con
Rosacea	Gravina et al[47] Argenziano et al[48] El-Khalawany et al[49]	
Psoriasis	Qayoom et al[52] Mesquita et al[53] Ribaldone et al[54] Onsun et al[55]	Campanati et al[50] Azizzadeh et al[51]
Chronic urticaria	Hizal et al[56] Galadari et al[57] Yoshimasu et al[59]	Campanati et al[58]
Alopecia areata	Behrangi et al[61]	Rigopoulos et al[60]
Autoimmune bullous diseases	Sagi et al[64] Mortazavi et al[65]	
Schöenlein-Henoch purpura	Novák et al[66] Grivceva-Panovska et al[67] Hoshino et al[68]	
Hematologic diseases	Pro	Con
Iron deficiency anemia	Blecker et al[69] Muhsen et al[70] Qu et al[71] Huang et al[72] Yuan et al[73] Zhang et al[74] Ozkasap et al[77] Senkovich et al[78] Yokota et al[79] Testerman et al[80] Afifi et al[81] Hershko et al[82] Hershko et al[83] Kang et al[84] Musumba et al[85] De Leest et al[86]	
Vitamin B12 deficiency	Campuzano-Maya et al[87] Lahner et al[88] Marino et al[90] Toh et al[91]	
Primary immune thrombocytopenia	García Pérez et al[92] Stasi et al[93] Gasbarrini et al[94] Suvajdzić et al[95] Jackson et al[96] Satoh et al[101] Asahi et al[102] Kodama et al[103] Byrne et al[104]	Jarque et al[97] Michel et al[98] Michel et al[99] Estrada-Gómez et al[100]
Autoimmune neutropenia	Cicconi et al[105] Papadaki et al[107] Papadaki et al[108]	
Plasma cell dyscrasias	Tursi et al[109] Wolkersdörfer et al[110]	Papadaki et al[107] Rajkumar et al[111]
Ocular diseases	Pro	Con
Open-angle glaucoma	Zeng J et al[114] Testerman et al[80]	Galloway et al[112] Kurtz et al[113]
Central serous chorioretinitis	Casella et al[115] Liu et al[116] Cotticelli et al[117] Rahbani-Nobar et al[118] Dang et al[119] Zavoloka et al[120]	
Blepharitis	Saccà et al[122]	Saccà et al[121]
Cardiovascular diseases	Pro	Con
Coronary atherosclerotic disease	Mendall et al[12] Lai et al[125] Chmiela et al[126] Chmiela et al[128] Chalubinski et al[129] Libby[130] Xu et al[131] Matsuura et al[136] Khodaii et al[137] Rasmi et al[138] Zhang et al[139]	Manolakis et al[141] Carter et al[142]
Myocardial infarction	Ikeda et al[143] Liu et al[144] Hughes et al[145]	
Metabolic disease	Pro	Con
Diabetes mellitus	Oldenburg et al[146] Hsieh et al[147] Bégué et al[148] Yang et al[150] Horikawa et al[151] Ibrahim et al[152] Chen et al[153] Jeon et al[156] Wang et al[158] Vafaeimanesh et al[160] Chung et al[161]	Demir et al[149] Akanuma et al[154] Yanik et al[159]
Insulin resistance syndrome	Vafaeimanesh et al[160] Chung et al[161] Zhou et al[162] Aydemir et al[163] Gunji et al[164] Eshraghian et al[165]	Naja et al[166]
Metabolic syndrome	Chen et al[167] Niemelä et al[168] Laurila et al[169] Polyzos et al[185]	

Allergic diseases	Pro	Con
		Chen *et al*[170]
		Wang *et al*[171]
		Zhou *et al*[172]
		Blaser *et al*[173]
		Amberbir *et al*[174]
		Oertli *et al*[175]
		Oertli *et al*[176]
		Cook *et al*[178]
		Engler *et al*[179]
		Grad *et al*[180]
		Sheikh *et al*[181]
		Molina-Infante *et al*[182]

by disturbing the balance between apoptosis and proliferation of hepatocytes. Senescence marker protein-30, a protein that prevents oxidative stress and hepatic cell apoptosis[194] is reduced in the liver of mice treated with CCL4 and *H. pylori* compared to those treated with CCL4 only[195].

H. pylori has been considered as putative a triggering factor for autoimmune extragastric conditions[196,197]. Goo *et al*[198] in 2008 showed that C57BL/6 mouse infected with *H. pylori* developed a form of primary biliary cirrhosis (PBC) very similar to that described in humans. Because of the high serum levels of IgG against *H. pylori* VacA, the authors suggested that anti-VacA antibodies might play a role in the development of PBC. Shapira *et al*[199] found higher prevalence of anti-*H. pylori* IgG in patients with PBC compared to controls. Nilsson *et al*[200] evaluated 24 liver biopsies obtained from patients with PBC and primary sclerosing cholangitis (PSC) and found that *H. pylori* was present in 20/24 patients. The authors a suggested that the pathogenic ling between *H. pylori* infection and autoimmune liver diseases might be a form of molecular mimicry between *H. pylori* and liver antigens. In fact, a similar amino acid sequence homology between the main mitochondrial autoepithopic region from the E2 subunit of the pyruvate dehydrogenase complex and the *H. pylori* urease was found. However Bogdanos *et al*[201] showed that the existence of this homology does not necessarily imply cross-reactivity. *H. pylori* DNA was detected in biliary epithelium in PSC patients compared to controls, corroborating the hypothesis that biliary reflux could lead to *H. pylori* contamination of the proximal biliary system, contributing to the development and / or progression of PSC in some patients[202]. Patients with PSC may also suffer from ulcerative colitis (UC). It has therefore been suggested that an increased intestinal permeability in UC patients may promote the translocation of *H. pylori* into the hepatobiliary system, thus triggering autoimmunity mechanisms[203].

The role of *H. pylori* in hepatic carcinogenesis is controversial. There are several known risk factors for HCC, such as alcoholism, PCB, PSC and chronic viral infections[204]. The finding of *H. pylori* in hepatic biopsies of patients with HCC led to the hypothesis that *H. pylori* might be playing a role also in the development of this infection[205]. In several studies, liver biopsies of

patients with HCC or even cholangiocarcinoma were positive for *H. pylori*[206,207]. Dore *et al*[208] demonstrated *H. pylori* presence in patients with HCC, liver cirrhosis and chronic hepatitis, using both PCR on liver tissue and serology. Prevalence of *H. pylori* infection was as high as 73% in patients with HCC. Verhoef *et al*[209] and Pellicano *et al*[210] also found positivity for *H. pylori* in 45% of patients with HCC *vs* 10% of controls, and in 85% of patients with HCC *vs* 33% of controls, respectively. Finally, it has been suggested that infection by CagA positive *H. pylori* strains might play a major role in the development of *H. pylori*-associated liver diseases[211].

To date, there is no sufficient evidence to draw a firm conclusion on the relationship between *H. pylori* infection and liver diseases.

CONCLUSION

H. pylori is the cause of a number of gastroduodenal diseases including peptic ulcer disease and gastric adenocarcinoma which are the results of an interaction between bacterial virulence factors, host and environmental factors. Many extra-gastric manifestations have been reported to be linked to *H. pylori* infection (Table 1). However, most evidences derive from epidemiological studies where a number of confounding factors have not been analyzed in depth, thus making it impossible to establish a cause-effect correlation. As a result of this, to date, according to the last Consensus Report on the management of *H. pylori* infection (*i.e.*, Maastricht V/Florence Guidelines[13]) *H. pylori* infection should be sought and, if present, eradicated only in patients with IDA, ITP and vitamin B12 deficiency.

REFERENCES

1 **Mégraud F**, Brassens-Rabbé MP, Denis F, Belbouri A, Hoa DQ. Seroepidemiology of Campylobacter pylori infection in various populations. *J Clin Microbiol* 1989; **27**: 1870-1873 [PMID: 2549098]
2 **Go MF**. Review article: natural history and epidemiology of Helicobacter pylori infection. *Aliment Pharmacol Ther* 2002; **16** Suppl 1: 3-15 [PMID: 11849122 DOI: 10.1046/j.1365-2036.2002.0160s1003.x]
3 **Leclerc H**. [Epidemiological aspects of Helicobacter pylori infection]. *Bull Acad Natl Med* 2006; **190**: 949-962 [PMID: 17195619]
4 **Mandeville KL**, Krabshuis J, Ladep NG, Mulder CJ, Quigley EM, Khan SA. Gastroenterology in developing countries: issues and advances. *World J Gastroenterol* 2009; **15**: 2839-2854 [PMID: 19533805 DOI: 10.3748/wjg.15.2839]
5 **Ahmed KS**, Khan AA, Ahmed I, Tiwari SK, Habeeb MA, Ali SM, Ahi JD, Abid Z, Alvi A, Hussain MA, Ahmed N, Habibullah CM. Prevalence study to elucidate the transmission pathways of Helicobacter pylori at oral and gastroduodenal sites of a South Indian population. *Singapore Med J* 2006; **47**: 291-296 [PMID: 16572240]
6 **Mladenova I**, Durazzo M. Transmission of Helicobacter pylori. *Minerva Gastroenterol Dietol* 2018; 64: 251-254 [PMID: 29458239 DOI: 10.23736/S1121-421X.18.02480-7]
7 **Blaser MJ**. Hypothesis: the changing relationships of Helicobacter pylori and humans: implications for health and disease. *J Infect Dis*

1999; **179**: 1523-1530 [PMID: 10228075 DOI: 10.1086/314785]

8   **Lehours P**, Mégraud F. Helicobacter pylori infection and gastric MALT lymphoma. *Rocz Akad Med Bialymst* 2005; **50**: 54-61 [PMID: 16358940]

9   **Romano M**, Ricci V, Zarrilli R. Mechanisms of disease: Helicobacter pylori-related gastric carcinogenesis--implications for chemoprevention. *Nat Clin Pract Gastroenterol Hepatol* 2006; **3**: 622-632 [PMID: 17068500 DOI: 10.1038/ncpgasthep0634]

10   **Censini S**, Stein M, Covacci A. Cellular responses induced after contact with Helicobacter pylori. *Curr Opin Microbiol* 2001; **4**: 41-46 [PMID: 11173032 DOI: 10.1016/S1369-5274(00)00162-4]

11   **El-Omar EM**, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF Jr, Rabkin CS. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* 2000; **404**: 398-402 [PMID: 10746728 DOI: 10.1038/35006081]

12   **Mendall MA**, Goggin PM, Molineaux N, Levy J, Toosy T, Strachan D, Camm AJ, Northfield TC. Relation of Helicobacter pylori infection and coronary heart disease. *Br Heart J* 1994; **71**: 437-439 [PMID: 8011406 DOI: 10.1136/hrt.71.5.437]

13   **Malfertheiner P**, Megraud F, O'Morain CA, Gisbert JP, Kuipers EJ, Axon AT, Bazzoli F, Gasbarrini A, Atherton J, Graham DY, Hunt R, Moayyedi P, Rokkas T, Rugge M, Selgrad M, Suerbaum S, Sugano K, El-Omar EM; European Helicobacter and Microbiota Study Group and Consensus panel. Management of Helicobacter pylori infection-the Maastricht V/Florence Consensus Report. *Gut* 2017; **66**: 6-30 [PMID: 27707777 DOI: 10.1136/gutjnl-2016-312288]

14   **Chen Y**, Segers S, Blaser MJ. Association between Helicobacter pylori and mortality in the NHANES III study. *Gut* 2013; **62**: 1262-1269 [PMID: 23303440 DOI: 10.1136/gutjnl-2012-303018]

15   **Wang ZW**, Li Y, Huang LY, Guan QK, Xu DW, Zhou WK, Zhang XZ. Helicobacter pylori infection contributes to high risk of ischemic stroke: evidence from a meta-analysis. *J Neurol* 2012; **259**: 2527-2537 [PMID: 22688569 DOI: 10.1007/s00415-012-6558-7]

16   **Alvarez-Arellano L**, Maldonado-Bernal C. Helicobacter pylori and neurological diseases: Married by the laws of inflammation. *World J Gastrointest Pathophysiol* 2014; **5**: 400-404 [PMID: 25400983 DOI: 10.4291/wjgp.v5.i4.400]

17   **Huang WS**, Yang TY, Shen WC, Lin CL, Lin MC, Kao CH. Association between Helicobacter pylori infection and dementia. *J Clin Neurosci* 2014; **21**: 1355-1358 [PMID: 24629396 DOI: 10.1016/j.jocn.2013.11.018]

18   **Roubaud Baudron C**, Letenneur L, Langlais A, Buissonnière A, Mégraud F, Dartigues JF, Salles N; Personnes Agées QUID Study. Does Helicobacter pylori infection increase incidence of dementia? The Personnes Agées QUID Study. *J Am Geriatr Soc* 2013; **61**: 74-78 [PMID: 23252507 DOI: 10.1111/jgs.12065]

19   **Beydoun MA**, Beydoun HA, Shroff MR, Kitner-Triolo MH, Zonderman AB. Helicobacter pylori seropositivity and cognitive performance among US adults: evidence from a large national survey. *Psychosom Med* 2013; **75**: 486-496 [PMID: 23697465 DOI: 10.1097/PSY.0b013e31829108c3]

20   **Goni E**, Franceschi F. Helicobacter pylori and extragastric diseases. *Helicobacter* 2016; **21** Suppl 1: 45-48 [PMID: 27531539 DOI: 10.1111/hel.12340]

21   **Kountouras J**, Boziki M, Gavalas E, Zavos C, Deretzi G, Chatzigeorgiou S, Katsinelos P, Grigoriadis N, Giartza-Taxidou E, Venizelos I. Five-year survival after Helicobacter pylori eradication in Alzheimer disease patients. *Cogn Behav Neurol* 2010; **23**: 199-204 [PMID: 20829670 DOI: 10.1097/WNN.0b013e3181df3034]

22   **Kountouras J**, Boziki M, Gavalas E, Zavos C, Grigoriadis N, Deretzi G, Tzilves D, Katsinelos P, Tsolaki M, Chatzopoulos D, Venizelos I. Eradication of Helicobacter pylori may be beneficial in the management of Alzheimer's disease. *J Neurol* 2009; **256**: 758-767 [PMID: 19240960 DOI: 10.1007/s00415-009-5011-z]

23   **Kountouras J**, Tsolaki F, Tsolaki M, Gavalas E, Zavos C, Polyzos SA, Boziki M, Katsinelos P, Kountouras C, Vardaka E, Tagarakis GI, Deretzi G. Helicobacter pylori-related ApoE 4 polymorphism

24   may be associated with dysphagic symptoms in older adults. *Dis Esophagus* 2016; **29**: 842 [PMID: 25873443 DOI: 10.1111/dote.12364]

24   **Santos CY**, Snyder PJ, Wu WC, Zhang M, Echeverria A, Alber J. Pathophysiologic relationship between Alzheimer's disease, cerebrovascular disease, and cardiovascular risk: A review and synthesis. *Alzheimers Dement* (Amst) 2017; **7**: 69-87 [PMID: 28275702 DOI: 10.1016/j.dadm.2017.01.005]

25   **Kountouras J**, Boziki M, Gavalas E, Zavos C, Deretzi G, Grigoriadis N, Tsolaki M, Chatzopoulos D, Katsinelos P, Tzilves D, Zabouri A, Michailidou I. Increased cerebrospinal fluid Helicobacter pylori antibody in Alzheimer's disease. *Int J Neurosci* 2009; **119**: 765-777 [PMID: 19326283 DOI: 10.1080/00207450902782083]

26   **Zelaya MV**, Pérez-Valderrama E, de Morentin XM, Tuñon T, Ferrer I, Luquin MR, Fernandez-Irigoyen J, Santamaría E. Olfactory bulb proteome dynamics during the progression of sporadic Alzheimer's disease: identification of common and distinct olfactory targets across Alzheimer-related co-pathologies. *Oncotarget* 2015; **6**: 39437-39456 [PMID: 26517091 DOI: 10.18632/oncotarget.6254]

27   **Attems J**, Walker L, Jellinger KA. Olfactory bulb involvement in neurodegenerative diseases. *Acta Neuropathol* 2014; **127**: 459-475 [PMID: 24554308 DOI: 10.1007/s00401-014-1261-7]

28   **Thomann PA**, Dos Santos V, Seidl U, Toro P, Essig M, Schröder J. MRI-derived atrophy of the olfactory bulb and tract in mild cognitive impairment and Alzheimer's disease. *J Alzheimers Dis* 2009; **17**: 213-221 [PMID: 19494444 DOI: 10.3233/JAD-2009-1036]

29   **Förster S**, Vaitl A, Teipel SJ, Yakushev I, Mustafa M, la Fougère C, Rominger A, Cumming P, Bartenstein P, Hampel H, Hummel T, Buerger K, Hundt W, Steinbach S. Functional representation of olfactory impairment in early Alzheimer's disease. *J Alzheimers Dis* 2010; **22**: 581-591 [PMID: 20847402 DOI: 10.3233/JAD-2010-091549]

30   **Doulberis M**, Kotronis G, Thomann R, Polyzos SA, Boziki M, Gialamprinou D, Deretzi G, Katsinelos P, Kountouras J. Review: Impact of Helicobacter pylori on Alzheimer's disease: What do we know so far? *Helicobacter* 2018; **23**: e12454 [PMID: 29181894 DOI: 10.1111/hel.12454]

31   **Shiota S**, Murakami K, Yoshiiwa A, Yamamoto K, Ohno S, Kuroda A, Mizukami K, Hanada K, Okimoto T, Kodama M, Abe K, Yamaoka Y, Fujioka T. The relationship between Helicobacter pylori infection and Alzheimer's disease in Japan. *J Neurol* 2011; **258**: 1460-1463 [PMID: 21336779 DOI: 10.1007/s00415-011-5957-5]

32   **Chang YP**, Chiu GF, Kuo FC, Lai CL, Yang YH, Hu HM, Chang PY, Chen CY, Wu DC, Yu FJ. Eradication of Helicobacter pylori Is Associated with the Progression of Dementia: A Population-Based Study. *Gastroenterol Res Pract* 2013; **2013**: 175729 [PMID: 24371435 DOI: 10.1155/2013/175729]

33   **Mohebi N**, Mamarabadi M, Moghaddasi M. Relation of helicobacter pylori infection and multiple sclerosis in Iranian patients. *Neurol Int* 2013; **5**: 31-33 [PMID: 23888213 DOI: 10.4081/ni.2013.e10]

34   **Franceschi F**, Gasbarrini A, Polyzos SA, Kountouras J. Extragastric Diseases and Helicobacter pylori. *Helicobacter* 2015; **20** Suppl 1: 40-46 [PMID: 26372824 DOI: 10.1111/hel.12256]

35   **Cook KW**, Crooks J, Hussain K, O'Brien K, Braitch M, Kareem H, Constantinescu CS, Robinson K, Gran B. Helicobacter pylori infection reduces disease severity in an experimental model of multiple sclerosis. *Front Microbiol* 2015; **6**: 52 [PMID: 25762984 DOI: 10.3389/fmicb.2015.00052]

36   **Shen X**, Yang H, Wu Y, Zhang D, Jiang H. Meta-analysis: Association of Helicobacter pylori infection with Parkinson's diseases. *Helicobacter* 2017; **22**: e12398 [PMID: 28598012 DOI: 10.1111/hel.12398]

37   **Huang HK**, Wang JH, Lei WY, Chen CL, Chang CY, Liou LS. Helicobacter pylori infection is associated with an increased risk of Parkinson's disease: A population-based retrospective cohort study. *Parkinsonism Relat Disord* 2018; **47**: 26-31 [PMID: 29174171 DOI: 10.1016/j.parkreldis.2017.11.331]

38   **Fasano A**, Bove F, Gabrielli M, Petracca M, Zocco MA, Ragazzoni E, Barbaro F, Piano C, Fortuna S, Tortora A, Di Giacopo R,

Campanale M, Gigante G, Lauritano EC, Navarra P, Marconi S, Gasbarrini A, Bentivoglio AR. The role of small intestinal bacterial overgrowth in Parkinson's disease. *Mov Disord* 2013; **28**: 1241-1249 [PMID: 23712625 DOI: 10.1002/mds.25522]

39 **Tan AH**, Mahadeva S, Marras C, Thalha AM, Kiew CK, Yeat CM, Ng SW, Ang SP, Chow SK, Loke MF, Vadivelu JS, Ibrahim N, Yong HS, Tan CT, Fox SH, Lang AE, Lim SY. Helicobacter pylori infection is associated with worse severity of Parkinson's disease. *Parkinsonism Relat Disord* 2015; **21**: 221-225 [PMID: 25560322 DOI: 10.1016/j.parkreldis.2014.12.009]

40 **Mridula KR**, Borgohain R, Chandrasekhar Reddy V, Bandaru VCh, Suryaprabha T. Association of Helicobacter pylori with Parkinson's Disease. *J Clin Neurol* 2017; **13**: 181-186 [PMID: 28406585 DOI: 10.3988/jcn.2017.13.2.181]

41 **Candelario-Jalil E**, Taheri S, Yang Y, Sood R, Grossetete M, Estrada EY, Fiebich BL, Rosenberg GA. Cyclooxygenase inhibition limits blood-brain barrier disruption following intracerebral injection of tumor necrosis factor-alpha in the rat. *J Pharmacol Exp Ther* 2007; **323**: 488-498 [PMID: 17704356 DOI: 10.1124/jpet.107.127035]

42 **Lo YC**, Shih YT, Wu DC, Lee YC. In vitro effects of Helicobacter pylori-induced infection in gastric epithelial AGS cells on microglia-mediated toxicity in neuroblastoma SH-SY5Y cells. *Inflamm Res* 2009; **58**: 329-335 [PMID: 19247579 DOI: 10.1007/s00011-009-8075-4]

43 **Dobbs RJ**, Charlett A, Purkiss AG, Dobbs SM, Weller C, Peterson DW. Association of circulating TNF-alpha and IL-6 with ageing and parkinsonism. *Acta Neurol Scand* 1999; **100**: 34-41 [PMID: 10416510 DOI: 10.1111/j.1600-0404.1999.tb00721.x]

44 **Kountouras J**, Deretzi G, Zavos C, Karatzoglou P, Touloumis L, Nicolaides T, Chatzopoulos D, Venizelos I. Association between Helicobacter pylori infection and acute inflammatory demyelinating polyradiculoneuropathy. *Eur J Neurol* 2005; **12**: 139-143 [PMID: 15679702 DOI: 10.1111/j.1468-1331.2004.00977.x]

45 **Moran AP**, Prendergast MM. Molecular mimicry in Campylobacter jejuni and Helicobacter pylori lipopolysaccharides: contribution of gastrointestinal infections to autoimmunity. *J Autoimmun* 2001; **16**: 241-256 [PMID: 11334489 DOI: 10.1006/jaut.2000.0490]

46 **Chiba S**, Sugiyama T, Yonekura K, Tanaka S, Matsumoto H, Fujii N, Ebisu S, Sekiguchi K. An antibody to VacA of Helicobacter pylori in cerebrospinal fluid from patients with Guillain-Barre syndrome. *J Neurol Neurosurg Psychiatry* 2002; **73**: 76-78 [PMID: 12082053 DOI: 10.1136/jnnp.73.1.76]

47 **Gravina A**, Federico A, Ruocco E, Lo Schiavo A, Masarone M, Tuccillo C, Peccerillo F, Miranda A, Romano L, de Sio C, de Sio I, Persico M, Ruocco V, Riegler G, Loguercio C, Romano M. Helicobacter pylori infection but not small intestinal bacterial overgrowth may play a pathogenic role in rosacea. *United European Gastroenterol J* 2015; **3**: 17-24 [PMID: 25653855 DOI: 10.1177/2050640614559262]

48 **Argenziano G**, Donnarumma G, Iovene MR, Arnese P, Baldassarre MA, Baroni A. Incidence of anti-Helicobacter pylori and anti-CagA antibodies in rosacea patients. *Int J Dermatol* 2003; **42**: 601-604 [PMID: 12890101 DOI: 10.1046/j.1365-4362.2003.01817.x]

49 **El-Khalawany M**, Mahmoud A, Mosbeh AS, A B D Alsalam F, Ghonaim N, Abou-Bakr A. Role of Helicobacter pylori in common rosacea subtypes: a genotypic comparative study of Egyptian patients. *J Dermatol* 2012; **39**: 989-995 [PMID: 23039081 DOI: 10.1111/j.1346-8138.2012.01675.x]

50 **Campanati A**, Ganzetti G, Martina E, Giannoni M, Gesuita R, Bendia E, Giuliodori K, Sandroni L, Offidani A. Helicobacter pylori infection in psoriasis: results of a clinical study and review of the literature. *Int J Dermatol* 2015; **54**: e109-e114 [PMID: 25808243 DOI: 10.1111/ijd.12798]

51 **Azizzadeh M**, Nejad ZV, Ghorbani R, Pahlevan D. Relationship between Helicobacter pylori infection and psoriasis. *Ann Saudi Med* 2014; **34**: 241-244 [PMID: 25266185 DOI: 10.5144/0256-4947.2014.241]

52 **Qayoom S**, Ahmad QM. Psoriasis and Helicobacter pylori. *Indian J Dermatol Venereol Leprol* 2003; **69**: 133-134 [PMID: 17642857]

53 **Mesquita PM**, Diogo A Filho, Jorge MT, Berbert AL, Mantese SA, Rodrigues JJ. Relationship of Helicobacter pylori seroprevalence with the occurrence and severity of psoriasis. *An Bras Dermatol* 2017; **92**: 52-57 [PMID: 28225957 DOI: 10.1590/abd1806-4841.20174880]

54 **Mesquita PMD**, Diogo A Filho, Jorge MT, Berbert ALCV, Mantese SAO, Rodrigues JJ. Comment on Helicobacter pylori seroprevalence and the occurrence and severity of psoriasis - Reply. *An Bras Dermatol* 2017; **92**: 585 [PMID: 28954123 DOI: 10.1590/abd1806-4841.20177256]

55 **Onsun N**, Arda Ulusal H, Su O, Beycan I, Biyik Ozkaya D, Senocak M. Impact of Helicobacter pylori infection on severity of psoriasis and response to treatment. *Eur J Dermatol* 2012; **22**: 117-120 [PMID: 22063790 DOI: 10.1684/ejd.2011.1579]

56 **Hizal M**, Tüzün B, Wolf R, Tüzün Y. The relationship between Helicobacter pylori IgG antibody and autologous serum test in chronic urticaria. *Int J Dermatol* 2000; **39**: 443-445 [PMID: 10944089 DOI: 10.1046/j.1365-4362.2000.00979.x]

57 **Galadari IH**, Sheriff MO. The role of Helicobacter pylori in urticaria and atopic dermatitis. *Skinmed* 2006; **5**: 172-176 [PMID: 16855407 DOI: 10.1111/j.1540-9740.2006.04646.x]

58 **Campanati A**, Gesuita R, Giannoni M, Piraccini F, Sandroni L, Martina E, Conocchiari L, Bendia E, Di Sario A, Offidani A. Role of small intestinal bacterial overgrowth and Helicobacter pylori infection in chronic spontaneous urticaria: a prospective analysis. *Acta Derm Venereol* 2013; **93**: 161-164 [PMID: 22858910 DOI: 10.2340/00015555-1373]

59 **Yoshimasu T**, Furukawa F. Eradication therapy for urticaria with high titers of anti H. pylori IgG antibody. *Allergol Int* 2014; **63**: 37-40 [PMID: 24270226 DOI: 10.2332/allergolint.13-OA-0580]

60 **Rigopoulos D**, Katsambas A, Karalexis A, Papatheodorou G, Rokkas T. No increased prevalence of Helicobacter pylori in patients with alopecia areata. *J Am Acad Dermatol* 2002; **46**: 141 [PMID: 11756964 DOI: 10.1067/mjd.2002.117255]

61 **Behrangi E**, Mansouri P, Agah S, Ebrahimi Daryani N, Mokhtare M, Azizi Z, Ramezani Ghamsari M, Rohani Nasab M, Azizian Z. Association between Helicobacter Pylori Infection and Alopecia Areata: A Study in Iranian Population. *Middle East J Dig Dis* 2017; **9**: 107-110 [PMID: 28638587 DOI: 10.15171/mejdd.2017.59]

62 **Ljubojevic S**, Lipozenčić J. Autoimmune bullous diseases associations. *Clin Dermatol* 2012; **30**: 17-33 [PMID: 22137223 DOI: 10.1016/j.clindermatol.2011.03.006]

63 **Magen E**, Delgado JS. Helicobacter pylori and skin autoimmune diseases. *World J Gastroenterol* 2014; **20**: 1510-1516 [PMID: 24587626 DOI: 10.3748/wjg.v20.i6.1510]

64 **Sagi L**, Baum S, Agmon-Levin N, Sherer Y, Katz BS, Barzilai O, Ram M, Bizzaro N, SanMarco M, Trau H, Shoenfeld Y. Autoimmune bullous diseases the spectrum of infectious agent antibodies and review of the literature. *Autoimmun Rev* 2011; **10**: 527-535 [PMID: 21527361 DOI: 10.1016/j.autrev.2011.04.003]

65 **Mortazavi H**, Hejazi P, Khamesipour A, Mohebali M, Ehsani AH, Mohammadi Y, Farahani IV, Amirzargar AA. Frequency of seropositivity against infectious agents amongst pemphigus vulgaris patients: a case-control study on Strongyloides stercoralis, Helicobacter pylori, Toxoplasma gondii, Leishmania major, and Epstein-Barr virus. *Int J Dermatol* 2015; **54**: e458-e465 [PMID: 26175264 DOI: 10.1111/ijd.12869]

66 **Novák J**, Szekanecz Z, Sebesi J, Takáts A, Demeter P, Bene L, Sipka S, Csiki Z. Elevated levels of anti-Helicobacter pylori antibodies in Henoch-Schönlein purpura. *Autoimmunity* 2003; **36**: 307-311 [PMID: 14567560 DOI: 10.1080/08916930232000114535]

67 **Grivceva-Panovska V**, Grivceva Stardelova K, Serafimoski V. Henoch-Schönlein purpura in an adult patient: extragastric, cutaneous manifestation of helicobacter pylori infection. *Prilozi* 2008; **29**: 291-301 [PMID: 18709017]

68 **Hoshino C**. Adult onset Schönlein-Henoch purpura associated with Helicobacter pylori infection. *Intern Med* 2009; **48**: 847-851 [PMID: 19443983 DOI: 10.2169/internalmedicine.48.1718]

69 **Blecker U**, Renders F, Lanciers S, Vandenplas Y. Syncopes leading to the diagnosis of a Helicobacter pylori positive chronic active

haemorrhagic gastritis. *Eur J Pediatr* 1991; **150**: 560-561 [PMID: 1954961 DOI: 10.1007/BF02072207]

70 **Muhsen K**, Cohen D. Helicobacter pylori infection and iron stores: a systematic review and meta-analysis. *Helicobacter* 2008; **13**: 323-340 [PMID: 19250507 DOI: 10.1111/j.1523-5378.2008.00617.x]

71 **Qu XH**, Huang XL, Xiong P, Zhu CY, Huang YL, Lu LG, Sun X, Rong L, Zhong L, Sun DY, Lin H, Cai MC, Chen ZW, Hu B, Wu LM, Jiang YB, Yan WL. Does Helicobacter pylori infection play a role in iron deficiency anemia? A meta-analysis. *World J Gastroenterol* 2010; **16**: 886-896 [PMID: 20143469 DOI: 10.3748/wjg.v16.i7.886]

72 **Huang X**, Qu X, Yan W, Huang Y, Cai M, Hu B, Wu L, Lin H, Chen Z, Zhu C, Lu L, Sun X, Rong L, Jiang Y, Sun D, Zhong L, Xiong P. Iron deficiency anaemia can be improved after eradication of Helicobacter pylori. *Postgrad Med J* 2010; **86**: 272-278 [PMID: 20448223 DOI: 10.1136/pgmj.2009.089987]

73 **Yuan W**, Li Yumin, Yang Kehu, Ma Bin, Guan Quanlin, Wang D, Yang L. Iron deficiency anemia in Helicobacter pylori infection: meta-analysis of randomized controlled trials. *Scand J Gastroenterol* 2010; **45**: 665-676 [PMID: 20201716 DOI: 10.3109/00365521003663670]

74 **Zhang ZF**, Yang N, Zhao G, Zhu L, Zhu Y, Wang LX. Effect of Helicobacter pylori eradication on iron deficiency. *Chin Med J* (Engl) 2010; **123**: 1924-1930 [PMID: 20819579]

75 **Azab SF**, Esh AM. Serum hepcidin levels in Helicobacter pylori-infected children with iron-deficiency anemia: a case-control study. *Ann Hematol* 2013; **92**: 1477-1483 [PMID: 23760782 DOI: 10.1007/s00277-013-1813-2]

76 **Kroot JJ**, Tjalsma H, Fleming RE, Swinkels DW. Hepcidin in human iron disorders: diagnostic implications. *Clin Chem* 2011; **57**: 1650-1669 [PMID: 21989113 DOI: 10.1373/clinchem.2009.140053]

77 **Ozkasap S**, Yarali N, Isik P, Bay A, Kara A, Tunc B. The role of prohepcidin in anemia due to Helicobacter pylori infection. *Pediatr Hematol Oncol* 2013; **30**: 425-431 [PMID: 23560993 DOI: 10.3109/08880018.2013.783144]

78 **Senkovich O**, Ceaser S, McGee DJ, Testerman TL. Unique host iron utilization mechanisms of Helicobacter pylori revealed with iron-deficient chemically defined media. *Infect Immun* 2010; **78**: 1841-1849 [PMID: 20176792 DOI: 10.1128/IAI.01258-09]

79 **Yokota S**, Toita N, Yamamoto S, Fujii N, Konno M. Positive relationship between a polymorphism in Helicobacter pylori neutrophil-activating protein a gene and iron-deficiency anemia. *Helicobacter* 2013; **18**: 112-116 [PMID: 23067298 DOI: 10.1111/hel.12011]

80 **Testerman TL**, Morris J. Beyond the stomach: an updated view of Helicobacter pylori pathogenesis, diagnosis, and treatment. *World J Gastroenterol* 2014; **20**: 12781-12808 [PMID: 25278678 DOI: 10.3748/wjg.v20.i36.12781]

81 **Afifi MT**, Abd El-Aziz HK, Hamed NA, Barghash NA, Abdo A, Gamal M. Role of Helicobacter pylori in refractory iron deficiency anaemia. *Br J Biomed Sci* 2009; **66**: 133-136 [PMID: 19839223 DOI: 10.1080/09674845.2009.11730259]

82 **Hershko C**, Camaschella C. How I treat unexplained refractory iron deficiency anemia. *Blood* 2014; **123**: 326-333 [PMID: 24215034 DOI: 10.1182/blood-2013-10-512624]

83 **Hershko C**, Ronson A. Iron deficiency, Helicobacter infection and gastritis. *Acta Haematol* 2009; **122**: 97-102 [PMID: 19907146 DOI: 10.1159/000243793]

84 **Kang JM**, Kim N, Lee BH, Park HK, Jo HJ, Shin CM, Lee SH, Park YS, Hwang JH, Kim JW, Jeong SH, Lee DH, Jung HC, Song IS. Risk factors for peptic ulcer bleeding in terms of Helicobacter pylori, NSAIDs, and antiplatelet agents. *Scand J Gastroenterol* 2011; **46**: 1295-1301 [PMID: 21815866 DOI: 10.3109/00365521.2011.605468]

85 **Musumba C**, Jorgensen A, Sutton L, Van Eker D, Moorcroft J, Hopkins M, Pritchard DM, Pirmohamed M. The relative contribution of NSAIDs and Helicobacter pylori to the aetiology of endoscopically-diagnosed peptic ulcer disease: observations from a

tertiary referral hospital in the UK between 2005 and 2010. *Aliment Pharmacol Ther* 2012; **36**: 48-56 [PMID: 22554233 DOI: 10.1111/j.1365-2036.2012.05118.x]

86 **De Leest HT**, Steen KS, Bloemena E, Lems WF, Kuipers EJ, Van de Laar MA, Bijlsma JW, Janssen M, Houben HH, Kostense PJ, Boers M, Dijkmans BA. Helicobacter pylori eradication in patients on long-term treatment with NSAIDs reduces the severity of gastritis: a randomized controlled trial. *J Clin Gastroenterol* 2009; **43**: 140-146 [PMID: 18797408 DOI: 10.1097/MCG.0b013e3181595b40]

87 **Campuzano-Maya G**. Hematologic manifestations of Helicobacter pylori infection. *World J Gastroenterol* 2014; **20**: 12818-12838 [PMID: 25278680 DOI: 10.3748/wjg.v20.i36.12818]

88 **Lahner E**, Persechino S, Annibale B. Micronutrients (Other than iron) and Helicobacter pylori infection: a systematic review. *Helicobacter* 2012; **17**: 1-15 [PMID: 22221610 DOI: 10.1111/j.1523-5378.2011.00892.x]

89 **Andrès E**, Loukili NH, Noel E, Kaltenbach G, Abdelgheni MB, Perrin AE, Noblet-Dick M, Maloisel F, Schlienger JL, Blicklé JF. Vitamin B12 (cobalamin) deficiency in elderly patients. *CMAJ* 2004; **171**: 251-259 [PMID: 15289425 DOI: 10.1503/cmaj.1031155]

90 **Marino MC**, de Oliveira CA, Rocha AM, Rocha GA, Clementino NC, Antunes LF, Oliveira RA, Martins AS, Del Puerto HL, D'Almeida V, Galdieri L, Pedroso ER, Cabral MM, Nogueira AM, Queiroz DM. Long-term effect of Helicobacter pylori eradication on plasma homocysteine in elderly patients with cobalamin deficiency. *Gut* 2007; **56**: 469-474 [PMID: 17005765 DOI: 10.1136/gut.2006.095125]

91 **Toh BH**, van Driel IR, Gleeson PA. Pernicious anemia. *N Engl J Med* 1997; **337**: 1441-1448 [PMID: 9358143 DOI: 10.1056/NEJM199711133372007]

92 **García Pérez A**, Valverde de La Osa J, Giménez Samper M, Alonso García I. Resolution of an autoimmune thrombocytopenic purpura after eradicating treatment of Helicobacter pylori. *Sangre* (Barc) 1999; **44**: 387-388 [PMID: 10618919]

93 **Stasi R**, Rossi Z, Stipa E, Amadori S, Newland AC, Provan D. Helicobacter pylori eradication in the management of patients with idiopathic thrombocytopenic purpura. *Am J Med* 2005; **118**: 414-419 [PMID: 15808140 DOI: 10.1016/j.amjmed.2004.09.014]

94 **Gasbarrini A**, Franceschi F, Tartaglione R, Landolfi R, Pola P, Gasbarrini G. Regression of autoimmune thrombocytopenia after eradication of Helicobacter pylori. *Lancet* 1998; **352**: 878 [PMID: 9742983 DOI: 10.1016/S0140-6736(05)60004-9]

95 **Suvajdzić N**, Stanković B, Artiko V, Cvejić T, Bulat V, Bakrac M, Colović M, Obradović V, Atkinson HD. Helicobacter pylori eradication can induce platelet recovery in chronic idiopathic thrombocytopenic purpura. *Platelets* 2006; **17**: 227-230 [PMID: 16769600 DOI: 10.1080/09537100500462487]

96 **Jackson SC**, Beck P, Buret AG, O'Connor PM, Meddings J, Pineo G, Poon MC. Long term platelet responses to Helicobacter pylori eradication in Canadian patients with immune thrombocytopenic purpura. *Int J Hematol* 2008; **88**: 212-218 [PMID: 18668306 DOI: 10.1007/s12185-008-0138-8]

97 **Jarque I**, Andreu R, Llopis I, De la Rubia J, Gomis F, Senent L, Jiménez C, Martín G, Martínez JA, Sanz GF, Ponce J, Sanz MA. Absence of platelet response after eradication of Helicobacter pylori infection in patients with chronic idiopathic thrombocytopenic purpura. *Br J Haematol* 2001; **115**: 1002-1003 [PMID: 11843840 DOI: 10.1046/j.1365-2141.2001.03194.x]

98 **Michel M**, Khellaf M, Desforges L, Lee K, Schaeffer A, Godeau B, Bierling P. Autoimmune thrombocytopenic Purpura and Helicobacter pylori infection. *Arch Intern Med* 2002; **162**: 1033-1036 [PMID: 11996614 DOI: 10.1001/archinte.162.9.1033]

99 **Michel M**, Cooper N, Jean C, Frissora C, Bussel JB. Does Helicobater pylori initiate or perpetuate immune thrombocytopenic purpura? *Blood* 2004; **103**: 890-896 [PMID: 12920031 DOI: 10.1182/blood-2003-03-0900]

100 **Estrada-Gómez RA**, Parra-Ortega I, Martínez-Barreda C, Ruiz-Argüelles GJ. Helicobacter pylori infection and thrombocytopenia:

a single-institution experience in Mexico. *Rev Invest Clin* 2007; **59**: 112-115 [PMID: 17633798]

101 **Satoh T**, Pandey JP, Okazaki Y, Asahi A, Kawakami Y, Ikeda Y, Kuwana M. Single nucleotide polymorphism of interleukin-1beta associated with Helicobacter pylori infection in immune thrombocytopenic purpura. *Tissue Antigens* 2009; **73**: 353-357 [PMID: 19317746 DOI: 10.1111/j.1399-0039.2009.01214.x]

102 **Asahi A**, Nishimoto T, Okazaki Y, Suzuki H, Masaoka T, Kawakami Y, Ikeda Y, Kuwana M. Helicobacter pylori eradication shifts monocyte Fcgamma receptor balance toward inhibitory FcgammaRIIB in immune thrombocytopenic purpura patients. *J Clin Invest* 2008; **118**: 2939-2949 [PMID: 18654664 DOI: 10.1172/JCI34496]

103 **Kodama M**, Kitadai Y, Ito M, Kai H, Masuda H, Tanaka S, Yoshihara M, Fujimura K, Chayama K. Immune response to CagA protein is associated with improved platelet count after Helicobacter pylori eradication in patients with idiopathic thrombocytopenic purpura. *Helicobacter* 2007; **12**: 36-42 [PMID: 17241299 DOI: 10.1111/j.1523-5378.2007.00477.x]

104 **Byrne MF**, Kerrigan SW, Corcoran PA, Atherton JC, Murray FE, Fitzgerald DJ, Cox DM. Helicobacter pylori binds von Willebrand factor and interacts with GPIb to induce platelet aggregation. *Gastroenterology* 2003; **124**: 1846-1854 [PMID: 12806618 DOI: 10.1016/S0016-5085(03)00397-4]

105 **Cicconi V**, Carloni E, Franceschi F, Nocente R, Silveri NG, Manna R, Servidei S, Bentivoglio AR, Gasbarrini A, Gasbarrini G. Disappearance of antiphospholipid antibodies syndrome after Helicobacter pylori eradication. *Am J Med* 2001; **111**: 163-164 [PMID: 11501549 DOI: 10.1016/S0002-9343(01)00738-0]

106 **Lim W**, Crowther MA, Eikelboom JW. Management of antiphospholipid antibody syndrome: a systematic review. *JAMA* 2006; **295**: 1050-1057 [PMID: 16507806 DOI: 10.1001/jama.295.9.1050]

107 **Papadaki HA**, Pontikoglou C, Stavroulaki E, Minadakis G, Eliopoulos DA, Pyrovolaki K, Skordilis P, Eliopoulos GD. High prevalence of Helicobacter pylori infection and monoclonal gammopathy of undetermined significance in patients with chronic idiopathic neutropenia. *Ann Hematol* 2005; **84**: 317-320 [PMID: 15731921 DOI: 10.1007/s00277-004-0996-y]

108 **Papadaki HA**, Pontikoglou C, Eliopoulos DG, Pyrovolaki K, Spyridaki R, Eliopoulos GD. Helicobacter pylori infection is probably the cause of chronic idiopathic neutropenia (CIN)-associated splenomegaly. *Am J Hematol* 2006; **81**: 142-144 [PMID: 16432851 DOI: 10.1002/ajh.20496]

109 **Tursi A**, Modeo ME. Monoclonal gammopathy of undetermined significance predisposing to Helicobacter pylori-related gastric mucosa-associated lymphoid tissue lymphoma. *J Clin Gastroenterol* 2002; **34**: 147-149 [PMID: 11782609 DOI: 10.1097/00004836-200202000-00009]

110 **Wolkersdörfer GW**, Haase M, Morgner A, Baretton G, Miehlke S. Monoclonal gammopathy of undetermined significance and Russell body formation in Helicobacter pylori gastritis. *Helicobacter* 2006; **11**: 506-510 [PMID: 16961813 DOI: 10.1111/j.1523-5378.2006.00443.x]

111 **Rajkumar SV**, Kyle RA, Plevak MF, Murray JA, Therneau TM. Helicobacter pylori infection and monoclonal gammopathy of undetermined significance. *Br J Haematol* 2002; **119**: 706-708 [PMID: 12437647 DOI: 10.1046/j.1365-2141.2002.03912.x]

112 **Galloway PH**, Warner SJ, Morshed MG, Mikelberg FS. Helicobacter pylori infection and the risk for open-angle glaucoma. *Ophthalmology* 2003; **110**: 922-925 [PMID: 12750090 DOI: 10.1016/S0161-6420(03)00093-9]

113 **Kurtz S**, Regenbogen M, Goldiner I, Horowitz N, Moshkowitz M. No association between Helicobacter pylori infection or CagA-bearing strains and glaucoma. *J Glaucoma* 2008; **17**: 223-226 [PMID: 18414109 DOI: 10.1097/IJG.0b013e31815a34ac]

114 **Zeng J**, Liu H, Liu X, Ding C. The Relationship Between Helicobacter pylori Infection and Open-Angle Glaucoma: A Meta-Analysis. *Invest Ophthalmol Vis Sci* 2015; **56**: 5238-5245 [PMID: 26258610 DOI: 10.1167/iovs.15-17059]

115 **Casella AM**, Berbel RF, Bressanim GL, Malaguido MR, Cardillo JA. Helicobacter pylori as a potential target for the treatment of central serous chorioretinopathy. *Clinics* (Sao Paulo) 2012; **67**: 1047-1052 [PMID: 23018302 DOI: 10.6061/clinics/2012(09)11]

116 **Liu B**, Deng T, Zhang J. RISK FACTORS FOR CENTRAL SEROUS CHORIORETINOPATHY: A Systematic Review and Meta-Analysis. *Retina* 2016; **36**: 9-19 [PMID: 26710181 DOI: 10.1097/IAE.0000000000000837]

117 **Cotticelli L**, Borrelli M, D'Alessio AC, Menzione M, Villani A, Piccolo G, Montella F, Iovene MR, Romano M. Central serous chorioretinopathy and Helicobacter pylori. *Eur J Ophthalmol* 2006; **16**: 274-278 [PMID: 16703546 DOI: 10.1177/112067210601600213]

118 **Rahbani-Nobar MB**, Javadzadeh A, Ghojazadeh L, Rafeey M, Ghorbanihaghjo A. The effect of Helicobacter pylori treatment on remission of idiopathic central serous chorioretinopathy. *Mol Vis* 2011; **17**: 99-103 [PMID: 21245962]

119 **Dang Y**, Mu Y, Zhao M, Li L, Guo Y, Zhu Y. The effect of eradicating Helicobacter pylori on idiopathic central serous chorioretinopathy patients. *Ther Clin Risk Manag* 2013; **9**: 355-360 [PMID: 24043941 DOI: 10.2147/TCRM.S50407]

120 **Zavoloka O**, Bezditko P, Lahorzhevska I, Zubkova D, Ilyina Y. Clinical efficiency of Helicobacter pylori eradication in the treatment of patients with acute central serous chorioretinopathy. *Graefes Arch Clin Exp Ophthalmol* 2016; **254**: 1737-1742 [PMID: 26979068 DOI: 10.1007/s00417-016-3315-0]

121 **Saccà SC**, Vagge A, Pulliero A, Izzotti A. Helicobacter pylori infection and eye diseases: a systematic review. *Medicine* (Baltimore) 2014; **93**: e216 [PMID: 25526440 DOI: 10.1097/MD.0000000000000216]

122 **Saccà SC**, Pascotto A, Venturino GM, Prigione G, Mastromarino A, Baldi F, Bilardi C, Savarino V, Brusati C, Rebora A. Prevalence and treatment of Helicobacter pylori in patients with blepharitis. *Invest Ophthalmol Vis Sci* 2006; **47**: 501-508 [PMID: 16431942 DOI: 10.1167/iovs.05-0323]

123 **Linz B**, Balloux F, Moodley Y, Manica A, Liu H, Roumagnac P, Falush D, Stamer C, Prugnolle F, van der Merwe SW, Yamaoka Y, Graham DY, Perez-Trallero E, Wadstrom T, Suerbaum S, Achtman M. An African origin for the intimate association between humans and Helicobacter pylori. *Nature* 2007; **445**: 915-918 [PMID: 17287725 DOI: 10.1038/nature05562]

124 **Marshall BJ**, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; **1**: 1311-1315 [PMID: 6145023 DOI: 10.1016/S0140-6736(84)91816-6]

125 **Lai CY**, Yang TY, Lin CL, Kao CH. Helicobacter pylori infection and the risk of acute coronary syndrome: a nationwide retrospective cohort study. *Eur J Clin Microbiol Infect Dis* 2015; **34**: 69-74 [PMID: 25063740 DOI: 10.1007/s10096-014-2207-7]

126 **Chmiela M**, Gajewski A, Rudnicka K. Helicobacter pylori vs coronary heart disease - searching for connections. *World J Cardiol* 2015; **7**: 187-203 [PMID: 25914788 DOI: 10.4330/wjc.v7.i4.187]

127 **Wroblewski LE**, Peek RM Jr. Targeted disruption of the epithelial-barrier by Helicobacter pylori. *Cell Commun Signal* 2011; **9**: 29 [PMID: 22044698 DOI: 10.1186/1478-811X-9-29]

128 **Chmiela M**, Miszczyk E, Rudnicka K. Structural modifications of Helicobacter pylori lipopolysaccharide: an idea for how to live in peace. *World J Gastroenterol* 2014; **20**: 9882-9897 [PMID: 25110419 DOI: 10.3748/wjg.v20.i29.9882]

129 **Chalubinski M**, Wojdan K, Dorantowicz R, Jackowska P, Gorzelak P, Broncel M. Comprehensive insight into immune regulatory mechanisms and vascular wall determinants of atherogenesis - emerging perspectives of immunomodulation. *Arch Med Sci* 2013; **9**: 159-165 [PMID: 23515919 DOI: 10.5114/aoms.2013.33355]

130 **Libby P**. Inflammation in atherosclerosis. *Nature* 2002; **420**: 868-874 [PMID: 12490960 DOI: 10.1038/nature01323]

131 **Xu XH**, Shah PK, Faure E, Equils O, Thomas L, Fishbein MC, Luthringer D, Xu XP, Rajavashisth TB, Yano J, Kaul S, Arditi M. Toll-like receptor-4 is expressed by macrophages in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL. *Circulation* 2001; **104**: 3103-3108 [PMID: 11748108 DOI: 10.1161/hc5001.100631]

132 **Li J**, Li JJ, Li Q, Li Z, Qian HY. A rational connection of inflammation with peripheral arterial disease. *Med Hypotheses* 2007; **69**: 1190-1195 [PMID: 17555883 DOI: 10.1016/j.mehy.2007.02.043]

133 **Libby P**, Ridker PM, Hansson GK; Leducq Transatlantic Network on Atherothrombosis. Inflammation in atherosclerosis: from pathophysiology to practice. *J Am Coll Cardiol* 2009; **54**: 2129-2138 [PMID: 19942084 DOI: 10.1016/j.jacc.2009.09.009]

134 **Perkov S,** Paro MMK, Vidjak V, Flegar-Mestric Z. The Evaluation of New Biomarkers of Inflammation and Angiogenesis in Peripheral Arterial Disease. In: Rezzani R, editor. Current trends in atherogenesis. London: IntechOpen, 2013: 97-120 [DOI: 10.5772/53341]

135 **Vahdat K**, Jafari SM, Pazoki R, Nabipour I. Concurrent increased high sensitivity C-reactive protein and chronic infections are associated with coronary artery disease: a population-based study. *Indian J Med Sci* 2007; **61**: 135-143 [PMID: 17337814 DOI: 10.4103/0019-5359.30748]

136 **Matsuura E**, Kobayashi K, Matsunami Y, Shen L, Quan N, Makarova M, Suchkov SV, Ayada K, Oguma K, Lopez LR. Autoimmunity, infectious immunity, and atherosclerosis. *J Clin Immunol* 2009; **29**: 714-721 [PMID: 19795194 DOI: 10.1007/s10875-009-9333-5]

137 **Khodaii Z**, Vakili H, Ghaderian SM, Najar RA, Panah AS. Association of Helicobacter pylori infection with acute myocardial infarction. *Coron Artery Dis* 2011; **22**: 6-11 [PMID: 20962628 DOI: 10.1097/MCA.0b013e3283402360]

138 **Rasmi Y**, Raeisi S, Seyyed Mohammadzad MH. Association of inflammation and cytotoxin-associated gene a positive strains of helicobacter pylori in cardiac syndrome x. *Helicobacter* 2012; **17**: 116-120 [PMID: 22404441 DOI: 10.1111/j.1523-5378.2011.00923.x]

139 **Zhang S**, Guo Y, Ma Y, Teng Y. Cytotoxin-associated gene-A-seropositive virulent strains of Helicobacter pylori and atherosclerotic diseases: a systematic review. *Chin Med J* (Engl) 2008; **121**: 946-951 [PMID: 18706211]

140 **Fischer W**, Prassl S, Haas R. Virulence mechanisms and persistence strategies of the human gastric pathogen Helicobacter pylori. *Curr Top Microbiol Immunol* 2009; **337**: 129-171 [PMID: 19812982 DOI: 10.1007/978-3-642-01846-6_5]

141 **Manolakis A**, Kapsoritakis AN, Potamianos SP. A review of the postulated mechanisms concerning the association of Helicobacter pylori with ischemic heart disease. *Helicobacter* 2007; **12**: 287-297 [PMID: 17669100 DOI: 10.1111/j.1523-5378.2007.00511.x]

142 **Carter AM**, Moayyedi P, Catto A, Heppell RM, Axon AT, Grant PJ. The influence of Helicobacter pylori status on circulating levels of the coagulation factors fibrinogen, von Willebrand factor, factor VII, and factor VIII. *Helicobacter* 1996; **1**: 65-69 [PMID: 9398916 DOI: 10.1111/j.1523-5378.1996.tb00011.x]

143 **Ikeda A**, Iso H, Sasazuki S, Inoue M, Tsugane S; JPHC Study Group. The combination of Helicobacter pylori- and cytotoxin-associated gene-A seropositivity in relation to the risk of myocardial infarction in middle-aged Japanese: The Japan Public Health Center-based study. *Atherosclerosis* 2013; **230**: 67-72 [PMID: 23958254 DOI: 10.1016/j.atherosclerosis.2013.06.013]

144 **Liu J**, Wang F, Shi S. Helicobacter pylori Infection Increase the Risk of Myocardial Infarction: A Meta-Analysis of 26 Studies Involving more than 20,000 Participants. *Helicobacter* 2015; **20**: 176-183 [PMID: 25382293 DOI: 10.1111/hel.12188]

145 **Hughes WS**. An hypothesis: the dramatic decline in heart attacks in the United States is temporally related to the decline in duodenal ulcer disease and Helicobacter pylori infection. *Helicobacter* 2014; **19**: 239-241 [PMID: 24689964 DOI: 10.1111/hel.12123]

146 **Oldenburg B**, Diepersloot RJ, Hoekstra JB. High seroprevalence of Helicobacter pylori in diabetes mellitus patients. *Dig Dis Sci* 1996; **41**: 458-461 [PMID: 8617115 DOI: 10.1007/BF02282318]

147 **Hsieh MC**, Wang SS, Hsieh YT, Kuo FC, Soon MS, Wu DC. Helicobacter pylori infection associated with high HbA1c and type 2 diabetes. *Eur J Clin Invest* 2013; **43**: 949-956 [PMID: 23879740 DOI: 10.1111/eci.12124]

148 **Bégué RE**, Gómez R, Compton T, Vargas A. Effect of Helicobacter pylori eradication in the glycemia of children with type 1 diabetes:

149 **Demir M**, Gokturk HS, Ozturk NA, Kulaksizoglu M, Serin E, Yilmaz U. Helicobacter pylori prevalence in diabetes mellitus patients with dyspeptic symptoms and its relationship to glycemic control and late complications. *Dig Dis Sci* 2008; **53**: 2646-2649 [PMID: 18320319 DOI: 10.1007/s10620-007-0185-7]

150 **Yang GH**, Wu JS, Yang YC, Huang YH, Lu FH, Chang CJ. Gastric Helicobacter pylori infection associated with risk of diabetes mellitus, but not prediabetes. *J Gastroenterol Hepatol* 2014; **29**: 1794-1799 [PMID: 24731067 DOI: 10.1111/jgh.12617]

151 **Horikawa C**, Kodama S, Fujihara K, Hirasawa R, Yachi Y, Suzuki A, Hanyu O, Shimano H, Sone H. High risk of failing eradication of Helicobacter pylori in patients with diabetes: a meta-analysis. *Diabetes Res Clin Pract* 2014; **106**: 81-87 [PMID: 25110103 DOI: 10.1016/j.diabres.2014.07.009]

152 **Ibrahim A**, Zaher T, Ghonemy TA, El-Azim SA, El-Azim MA, Ramadan A. Impact of cytotoxin-associated gene A of Helicobacter pylori strains on microalbuminuria in type 2 diabetes. *Saudi J Kidney Dis Transpl* 2010; **21**: 694-700 [PMID: 20587874]

153 **Chen Y**, Blaser MJ. Association between gastric Helicobacter pylori colonization and glycated hemoglobin levels. *J Infect Dis* 2012; **205**: 1195-1202 [PMID: 22427676 DOI: 10.1093/infdis/jis106]

154 **Akanuma M**, Yanai A, Sakamoto K, Hirata Y, Yamaji Y, Kawazu S, Maeda S. Influence of Helicobacter pylori eradication on the management of type 2 diabetes. *Hepatogastroenterology* 2012; **59**: 641-645 [PMID: 22328266 DOI: 10.5754/hge11960]

155 **Wang X**, Bao W, Liu J, Ouyang YY, Wang D, Rong S, Xiao X, Shan ZL, Zhang Y, Yao P, Liu LG. Inflammatory markers and risk of type 2 diabetes: a systematic review and meta-analysis. *Diabetes Care* 2013; **36**: 166-175 [PMID: 23264288 DOI: 10.2337/dc12-0702]

156 **Jeon CY**, Haan MN, Cheng C, Clayton ER, Mayeda ER, Miller JW, Aiello AE. Helicobacter pylori infection is associated with an increased rate of diabetes. *Diabetes Care* 2012; **35**: 520-525 [PMID: 22279028 DOI: 10.2337/dc11-1043]

157 **Calle MC**, Fernandez ML. Inflammation and type 2 diabetes. *Diabetes Metab* 2012; **38**: 183-191 [PMID: 22252015 DOI: 10.1016/j.diabet.2011.11.006]

158 **Wang F**, Fu Y, Lv Z. Association of Helicobacter pylori infection with diabetic complications: a meta-analysis. *Endocr Res* 2014; **39**: 7-12 [PMID: 23879556 DOI: 10.3109/07435800.2013.794426]

159 **Yanik S**, Doğan Z, Sarikaya M, Ergul B, Filik L. Helicobacter pylori eradication reduces microalbuminuria in type-2 diabetic patients: a prospective study. *Acta Gastroenterol Belg* 2014; **77**: 235-239 [PMID: 25090822]

160 **Vafaeimanesh J**, Parham M, Seyyedmajidi M, Bagherzadeh M. Helicobacter pylori infection and insulin resistance in diabetic and nondiabetic population. *ScientificWorldJournal* 2014; **2014**: 391250 [PMID: 25405220 DOI: 10.1155/2014/391250]

161 **Chung GE**, Heo NJ, Park MJ, Chung SJ, Kang HY, Kang SJ. Helicobacter pylori seropositivity in diabetic patients is associated with microalbuminuria. *World J Gastroenterol* 2013; **19**: 97-102 [PMID: 23326169 DOI: 10.3748/wjg.v19.i1.97]

162 **Zhou X**, Liu W, Gu M, Zhou H, Zhang G. Helicobacter pylori infection causes hepatic insulin resistance by the c-Jun/miR-203/SOCS3 signaling pathway. *J Gastroenterol* 2015; **50**: 1027-1040 [PMID: 25689935 DOI: 10.1007/s00535-015-1051-6]

163 **Aydemir S**, Bayraktaroglu T, Sert M, Sokmen C, Atmaca H, Mungan G, Gun BD, Borazan A, Ustundag Y. The effect of Helicobacter pylori on insulin resistance. *Dig Dis Sci* 2005; **50**: 2090-2093 [PMID: 16240220 DOI: 10.1007/s10620-005-3012-z]

164 **Gunji T**, Matsuhashi N, Sato H, Fujibayashi K, Okumura M, Sasabe N, Urabe A. Helicobacter pylori infection significantly increases insulin resistance in the asymptomatic Japanese population. *Helicobacter* 2009; **14**: 144-150 [PMID: 19751440 DOI: 10.1111/j.1523-5378.2009.00705.x]

165 **Eshraghian A**, Hashemi SA, Hamidian Jahromi A, Eshraghian H, Masoompour SM, Davarpanah MA, Eshraghian K, Taghavi SA.

a preliminary study. *South Med J* 2002; **95**: 842-845 [PMID: 12190218 DOI: 10.1097/00007611-200295080-00012]

Helicobacter pylori infection as a risk factor for insulin resistance. *Dig Dis Sci* 2009; **54**: 1966-1970 [PMID: 19009348 DOI: 10.1007/s10620-008-0557-7]

166 **Naja F**, Nasreddine L, Hwalla N, Moghames P, Shoaib H, Fatfat M, Sibai A, Gali-Muhtasib H. Association of H. pylori infection with insulin resistance and metabolic syndrome among Lebanese adults. *Helicobacter* 2012; **17**: 444-451 [PMID: 23066847 DOI: 10.1111/j.1523-5378.2012.00970.x]

167 **Chen TP**, Hung HF, Chen MK, Lai HH, Hsu WF, Huang KC, Yang KC. Helicobacter Pylori Infection is Positively Associated with Metabolic Syndrome in Taiwanese Adults: a Cross-Sectional Study. *Helicobacter* 2015; **20**: 184-191 [PMID: 25582223 DOI: 10.1111/hel.12190]

168 **Niemelä S**, Karttunen T, Korhonen T, Läärä E, Karttunen R, Ikäheimo M, Kesäniemi YA. Could Helicobacter pylori infection increase the risk of coronary heart disease by modifying serum lipid concentrations? *Heart* 1996; **75**: 573-575 [PMID: 8697159 DOI: 10.1136/hrt.75.6.573]

169 **Laurila A**, Bloigu A, Näyhä S, Hassi J, Leinonen M, Saikku P. Association of Helicobacter pylori infection with elevated serum lipids. *Atherosclerosis* 1999; **142**: 207-210 [PMID: 9920523 DOI: 10.1016/S0021-9150(98)00194-4]

170 **Chen Y**, Blaser MJ. Inverse associations of Helicobacter pylori with asthma and allergy. *Arch Intern Med* 2007; **167**: 821-827 [PMID: 17452546 DOI: 10.1001/archinte.167.8.821]

171 **Wang Q**, Yu C, Sun Y. The association between asthma and Helicobacter pylori: a meta-analysis. *Helicobacter* 2013; **18**: 41-53 [PMID: 23067334 DOI: 10.1111/hel.12012]

172 **Zhou X**, Wu J, Zhang G. Association between Helicobacter pylori and asthma: a meta-analysis. *Eur J Gastroenterol Hepatol* 2013; **25**: 460-468 [PMID: 23242126 DOI: 10.1097/MEG.0b013e32835c280a]

173 **Blaser MJ**, Chen Y, Reibman J. Does Helicobacter pylori protect against asthma and allergy? *Gut* 2008; **57**: 561-567 [PMID: 18194986 DOI: 10.1136/gut.2007.133462]

174 **Amberbir A**, Medhin G, Erku W, Alem A, Simms R, Robinson K, Fogarty A, Britton J, Venn A, Davey G. Effects of Helicobacter pylori, geohelminth infection and selected commensal bacteria on the risk of allergic disease and sensitization in 3-year-old Ethiopian children. *Clin Exp Allergy* 2011; **41**: 1422-1430 [PMID: 21831135 DOI: 10.1111/j.1365-2222.2011.03831.x]

175 **Oertli M**, Sundquist M, Hitzler I, Engler DB, Arnold IC, Reuter S, Maxeiner J, Hansson M, Taube C, Quiding-Järbrink M, Müller A. DC-derived IL-18 drives Treg differentiation, murine Helicobacter pylori-specific immune tolerance, and asthma protection. *J Clin Invest* 2012; **122**: 1082-1096 [PMID: 22307326 DOI: 10.1172/JCI61029]

176 **Oertli M**, Noben M, Engler DB, Semper RP, Reuter S, Maxeiner J, Gerhard M, Taube C, Müller A. Helicobacter pylori γ-glutamyl transpeptidase and vacuolating cytotoxin promote gastric persistence and immune tolerance. *Proc Natl Acad Sci USA* 2013; **110**: 3047-3052 [PMID: 23382221 DOI: 10.1073/pnas.1211248110]

177 **Memon AA**, Hussein NR, Miendje Deyi VY, Burette A, Atherton JC. Vacuolating cytotoxin genotypes are strong markers of gastric cancer and duodenal ulcer-associated Helicobacter pylori strains: a matched case-control study. *J Clin Microbiol* 2014; **52**: 2984-2989 [PMID: 24920772 DOI: 10.1128/JCM.00551-14]

178 **Cook KW**, Letley DP, Ingram RJ, Staples E, Skjoldmose H, Atherton JC, Robinson K. CCL20/CCR6-mediated migration of regulatory T cells to the Helicobacter pylori-infected human gastric mucosa. *Gut* 2014; **63**: 1550-1559 [PMID: 24436142 DOI: 10.1136/gutjnl-2013-306253]

179 **Engler DB**, Reuter S, van Wijck Y, Urban S, Kyburz A, Maxeiner J, Martin H, Yogev N, Waisman A, Gerhard M, Cover TL, Taube C, Müller A. Effective treatment of allergic airway inflammation with Helicobacter pylori immunomodulators requires BATF3-dependent dendritic cells and IL-10. *Proc Natl Acad Sci USA* 2014; **111**: 11810-11815 [PMID: 25074917 DOI: 10.1073/pnas.1410579111]

180 **Grad YH**, Lipsitch M, Aiello AE. Secular trends in Helicobacter pylori seroprevalence in adults in the United States: evidence for sustained race/ethnic disparities. *Am J Epidemiol* 2012; **175**: 54-59 [PMID: 22085628 DOI: 10.1093/aje/kwr288]

181 **Sheikh A**, Strachan DP. The hygiene theory: fact or fiction? *Curr Opin Otolaryngol Head Neck Surg* 2004; **12**: 232-236 [PMID: 15167035 DOI: 10.1097/01.moo.0000122311.13359.30]

182 **Molina-Infante J**, Gutierrez-Junquera C, Savarino E, Penagini R, Modolell I, Bartolo O, Prieto-García A, Mauro A, Alcedo J, Perelló A, Guarner-Argente C, Alcaide N, Vegas AM, Barros-García P, Murzi-Pulgar M, Perona M, Gisbert JP, Lucendo AJ; Upper GI Tract Study Group from the Spanish Gastroenterological Association (AEG). Helicobacter pylori infection does not protect against eosinophilic esophagitis: results from a large multicenter case-control study. *Am J Gastroenterol* 2018; [Epub ahead of print] [PMID: 29545632 DOI: 10.1038/s41395-018-0035-6]

183 **Doğan Z**, Filik L, Ergül B, Sarikaya M, Akbal E. Association between Helicobacter pylori and liver-to-spleen ratio: a randomized-controlled single-blind study. *Eur J Gastroenterol Hepatol* 2013; **25**: 107-110 [PMID: 23013624 DOI: 10.1097/MEG.0b013e3283590c10]

184 **Waluga M**, Kukla M, Żorniak M, Bacik A, Kotulski R. From the stomach to other organs: Helicobacter pylori and the liver. *World J Hepatol* 2015; **7**: 2136-2146 [PMID: 26328025 DOI: 10.4254/wjh.v7.i18.2136]

185 **Polyzos SA**, Kountouras J, Zavos C, Deretzi G. The association between Helicobacter pylori infection and insulin resistance: a systematic review. *Helicobacter* 2011; **16**: 79-88 [PMID: 21435084 DOI: 10.1111/j.1523-5378.2011.00822.x]

186 **Chen CX**, Mao YS, Foster P, Zhu ZW, Du J, Guo CY. Possible association between Helicobacter pylori infection and nonalcoholic fatty liver disease. *Appl Physiol Nutr Metab* 2017; **42**: 295-301 [PMID: 28177748 DOI: 10.1139/apnm-2016-0499]

187 **Boutari C**, Perakakis N, Mantzoros CS. Association of Adipokines with Development and Progression of Nonalcoholic Fatty Liver Disease. *Endocrinol Metab* (Seoul) 2018; **33**: 33-43 [PMID: 29589386 DOI: 10.3803/EnM.2018.33.1.33]

188 **Fukuda Y**, Bamba H, Okui M, Tamura K, Tanida N, Satomi M, Shimoyama T, Nishigami T. Helicobacter pylori infection increases mucosal permeability of the stomach and intestine. *Digestion* 2001; **63** Suppl 1: 93-96 [PMID: 11173917 DOI: 10.1159/000051918]

189 **Sumida Y**, Kanemasa K, Imai S, Mori K, Tanaka S, Shimokobe H, Kitamura Y, Fukumoto K, Kakutani A, Ohno T, Taketani H, Seko Y, Ishiba H, Hara T, Okajima A, Yamaguchi K, Moriguchi M, Mitsuyoshi H, Yasui K, Minami M, Itoh Y. Helicobacter pylori infection might have a potential role in hepatocyte ballooning in nonalcoholic fatty liver disease. *J Gastroenterol* 2015; **50**: 996-1004 [PMID: 25622927 DOI: 10.1007/s00535-015-1039-2]

190 **Basso D**, Plebani M, Kusters JG. Pathogenesis of Helicobacter pylori infection. *Helicobacter* 2010; **15** Suppl 1: 14-20 [PMID: 21054648 DOI: 10.1111/j.1523-5378.2010.00781.x]

191 **Hotamisligil GS**, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* 1996; **271**: 665-668 [PMID: 8571133]

192 **Ohashi K**, Parker JL, Ouchi N, Higuchi A, Vita JA, Gokce N, Pedersen AA, Kalthoff C, Tullin S, Sams A, Summer R, Walsh K. Adiponectin promotes macrophage polarization toward an anti-inflammatory phenotype. *J Biol Chem* 2010; **285**: 6153-6160 [PMID: 20028977 DOI: 10.1074/jbc.M109.088708]

193 **Ki MR**, Goo MJ, Park JK, Hong IH, Ji AR, Han SY, You SY, Lee EM, Kim AY, Park SJ, Lee HJ, Kim SY, Jeong KS. Helicobacter pylori accelerates hepatic fibrosis by sensitizing transforming growth factor-β1-induced inflammatory signaling. *Lab Invest* 2010; **90**: 1507-1516 [PMID: 20531291 DOI: 10.1038/labinvest.2010.109]

194 **Lee A**, O'Rourke J, De Ungria MC, Robertson B, Daskalopoulos G, Dixon MF. A standardized mouse model of Helicobacter pylori infection: introducing the Sydney strain. *Gastroenterology* 1997; **112**: 1386-1397 [PMID: 9098027 DOI: 10.1016/S0016-5085(97)70155-0]

195 **Goo MJ**, Ki MR, Lee HR, Yang HJ, Yuan DW, Hong IH, Park JK, Hong KS, Han JY, Hwang OK, Kim DH, Do SH, Cohn RD, Jeong

KS. Helicobacter pylori promotes hepatic fibrosis in the animal model. *Lab Invest* 2009; **89**: 1291-1303 [PMID: 19736546 DOI: 10.1038/labinvest.2009.90]

196   **Smyk DS**, Koutsoumpas AL, Mytilinaiou MG, Rigopoulou EI, Sakkas LI, Bogdanos DP. Helicobacter pylori and autoimmune disease: cause or bystander. *World J Gastroenterol* 2014; **20**: 613-629 [PMID: 24574735 DOI: 10.3748/wjg.v20.i3.613]

197   **Hasni S**, Ippolito A, Illei GG. Helicobacter pylori and autoimmune diseases. *Oral Dis* 2011; **17**: 621-627 [PMID: 21902767 DOI: 10.1111/j.1601-0825.2011.01796.x]

198   **Goo MJ**, Ki MR, Lee HR, Hong IH, Park JK, Yang HJ, Yuan DW, Hwang OK, Do SH, Yoo SE, Jeong KS. Primary biliary cirrhosis, similar to that in human beings, in a male C57BL/6 mouse infected with Helicobacter pylori. *Eur J Gastroenterol Hepatol* 2008; **20**: 1045-1048 [PMID: 18787477 DOI: 10.1097/MEG.0b013e3282f5e9db]

199   **Shapira Y**, Agmon-Levin N, Renaudineau Y, Porat-Katz BS, Barzilai O, Ram M, Youinou P, Shoenfeld Y. Serum markers of infections in patients with primary biliary cirrhosis: evidence of infection burden. *Exp Mol Pathol* 2012; **93**: 386-390 [PMID: 23022373 DOI: 10.1016/j.yexmp.2012.09.012]

200   **Nilsson HO**, Taneera J, Castedal M, Glatz E, Olsson R, Wadström T. Identification of Helicobacter pylori and other Helicobacter species by PCR, hybridization, and partial DNA sequencing in human liver samples from patients with primary sclerosing cholangitis or primary biliary cirrhosis. *J Clin Microbiol* 2000; **38**: 1072-1076 [PMID: 10698999]

201   **Bogdanos DP**, Baum H, Gunsar F, Arioli D, Polymeros D, Ma Y, Burroughs AK, Vergani D. Extensive homology between the major immunodominant mitochondrial antigen in primary biliary cirrhosis and Helicobacter pylori does not lead to immunological cross-reactivity. *Scand J Gastroenterol* 2004; **39**: 981-987 [PMID: 15513338 DOI: 10.1080/00365520410003236]

202   **Krasinskas AM**, Yao Y, Randhawa P, Dore MP, Sepulveda AR. Helicobacter pylori may play a contributory role in the pathogenesis of primary sclerosing cholangitis. *Dig Dis Sci* 2007; **52**: 2265-2270 [PMID: 17393314 DOI: 10.1007/s10620-007-9803-7]

203   **Casswall TH**, Németh A, Nilsson I, Wadström T, Nilsson HO. Helicobacter species DNA in liver and gastric tissues in children and adolescents with chronic liver disease. *Scand J Gastroenterol* 2010; **45**: 160-167 [PMID: 20095882 DOI: 10.3109/00365520903426915]

204   **Rabelo-Gonçalves EM**, Roesler BM, Zeitune JM. Extragastric manifestations of Helicobacter pylori infection: Possible role of bacterium in liver and pancreas diseases. *World J Hepatol* 2015; **7**: 2968-2979 [PMID: 26730276 DOI: 10.4254/wjh.v7.i30.2968]

205   **Tu QV**, Okoli AS, Kovach Z, Mendz GL. Hepatocellular carcinoma: prevalence and molecular pathogenesis of Helicobacter spp. *Future Microbiol* 2009; **4**: 1283-1301 [PMID: 19995189 DOI: 10.2217/fmb.09.90]

206   **Avenaud P**, Marais A, Monteiro L, Le Bail B, Bioulac Sage P, Balabaud C, Mégraud F. Detection of Helicobacter species in the liver of patients with and without primary liver carcinoma. *Cancer* 2000; **89**: 1431-1439 [PMID: 11013355 DOI: 10.1002/1097-0142(20001001)89:73.0.CO;2-5]

207   **Nilsson HO**, Mulchandani R, Tranberg KG, Stenram U, Wadström T. Helicobacter species identified in liver from patients with cholangiocarcinoma and hepatocellular carcinoma. *Gastroenterology* 2001; **120**: 323-324 [PMID: 11246512 DOI: 10.1053/gast.2001.21382]

208   **Dore MP**, Realdi G, Mura D, Graham DY, Sepulveda AR. Helicobacter infection in patients with HCV-related chronic hepatitis, cirrhosis, and hepatocellular carcinoma. *Dig Dis Sci* 2002; **47**: 1638-1643 [PMID: 12141829 DOI: 10.1023/A:1015848009444]

209   **Verhoef C**, Pot RG, de Man RA, Zondervan PE, Kuipers EJ, IJzermans JN, Kusters JG. Detection of identical Helicobacter DNA in the stomach and in the non-cirrhotic liver of patients with hepatocellular carcinoma. *Eur J Gastroenterol Hepatol* 2003; **15**: 1171-1174 [PMID: 14560149 DOI: 10.1097/00042737-200311000-00004]

210   **Pellicano R**, Mazzaferro V, Grigioni WF, Cutufia MA, Fagoonee S, Silengo L, Rizzetto M, Ponzetto A. Helicobacter species sequences in liver samples from patients with and without hepatocellular carcinoma. *World J Gastroenterol* 2004; **10**: 598-601 [PMID: 14966925 DOI: 10.3748/wjg.v10.i4.598]

211   **Esmat G**, El-Bendary M, Zakarya S, Ela MA, Zalata K. Role of Helicobacter pylori in patients with HCV-related chronic hepatitis and cirrhosis with or without hepatocellular carcinoma: possible association with disease progression. *J Viral Hepat* 2012; **19**: 473-479 [PMID: 22676359 DOI: 10.1111/j.1365-2893.2011.01567.x]

REVIEW

ATP-binding cassette transporters in progression and clinical outcome of pancreatic cancer: What is the way forward?

Aleksandra Adamska, Marco Falasca

Aleksandra Adamska, Marco Falasca, Metabolic Signalling Group, School of Pharmacy and Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Perth 6102, WA, Australia

ORCID number: Aleksandra Adamska (0000-0002-7152-4149); Marco Falasca (0000-0002-9801-7235).

Correspondence to: Marco Falasca, PhD, Professor, Metabolic Signalling Group, School of Pharmacy and Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, GPO Box U1987, Perth 6102, WA, Australia. marco.falasca@curtin.edu.au
Telephone: +61-8-92669712

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive diseases and is characterized by high chemoresistance, leading to the lack of effective therapeutic approaches and grim prognosis. Despite increasing understanding of the mechanisms of chemoresistance in cancer and the role of ATP-binding cassette (ABC) transporters in this resistance, the therapeutic potential of their pharmacological inhibition has not been successfully exploited yet. In spite of the discovery of potent pharmacological modulators of ABC transporters, the results obtained in clinical trials have been so far disappointing, with high toxicity levels impairing their successful administration to the patients. Critically, although ABC transporters have been mostly studied for their involvement in development of multidrug resistance (MDR), in recent years the contribution of ABC transporters to cancer initiation and progression has emerged as an important area of research, the understanding of which could significantly influence the development of more specific and efficient therapies. In this review, we explore the role of ABC transporters in the development and progression of malignancies, with focus on PDAC. Their established involvement in development of MDR will be also presented. Moreover, an emerging role for ABC transporters as prognostic tools for patients' survival will be discussed, demonstrating the therapeutic potential of ABC transporters in cancer therapy.

Key words: Pancreatic ductal adenocarcinoma; Multi-drug resistance; ATP-binding cassette transporters; Targeted therapies; Pancreatic ductal adenocarcinoma prognosis; Predictive markers

Core tip: Pancreatic cancer is one of the deadliest cancers due to its highly aggressive biology and resistance to broad range of therapeutics. Expression of ATP-binding cassette (ABC) transporters by cancer cells is one of the main mechanisms responsible for the lowered drug accumulation. However, the attempts made in multidrug resistance reversal by the inhibition of their activity have not provided satisfactory results in clinical trials. Nevertheless, current knowledge on the role played by ABC transporters in carcinogenesis beyond chemoresistance, could create the opportunity for the development of novel, direct targeted therapeutic strategies. Additionally, the association between ABC transporters expression and pancreatic ductal adenocarcinoma patients' prognosis and response to applied therapies confirms their pharmacological potential.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most fatal diseases in western world. Although not one of the leading causes of death, PDAC is certainly to be considered amid the most unfavourable cancers, ranking at 4th place in terms of death rate, with a 7%-8% chance of 5-year survival in United States[1]. Despite the progress made in understanding the biology and in the treatment of different cancer types, the mortality of PDAC patients still nearly equals its incidence and has not changed remarkably for the last few decades. The dismal prognosis of PDAC is the result of multiple factors including an aggressive nature, chemo- and radio-resistance and the lack of effective treatments and diagnostic tools. Therefore, when diagnosed, the vast majority of PDAC patients present with metastatic disease, not susceptible for surgery[2]. Only one fifth of the patients have the tumour resected and, unfortunately, most of them eventually relapse. Post-operative chemo- and radiotherapy are usually applied in order to delay tumour recurrence; nevertheless, high resistance and the heterogeneous nature of pancreatic tumours impede its treatment[3].

Pancreatic cancer pathology is a multistep process. It arises as an accumulation of abnormalities, both genetic and physiological, progressing through 3 stages of precursor lesions called pancreatic intraepithelial neoplasias (PanINs) before transforming into a fully differentiated tumour[4]. The substantial number of genetic modifications and consequent dysregulation of the wide range of essential signalling pathways accompanying these processes make PDAC highly heterogeneous[5]. Also, the variability of mutations between patients as well as within the same tumour contributes to its high resistance to applied therapy. High heterogeneity of PDAC is expressed also phenotypically. Genetically diverse subclones, possessing different metabolic and functional characteristics, exist within a tumour. Recent evidence shows that one of the populations acquires characteristics similar to stem cells, which enables it to survive during stressful conditions and is partly responsible for cancer relapse after treatment[6]. Furthermore, PDAC cell plasticity, which plays a role in epithelial to mesenchymal transition (EMT), facilitates metastatic spread and adds to the dismal prognosis[7]. Moreover, one of the main characteristics of PDAC, responsible for therapies' failure, is the formation of dense desmoplastic reaction, influencing cancer progression and impeding drug delivery to the tumour[8]. The interplay between tumour cells and stromal components (pancreatic stellate cells (PSCs), immune cells, cytokines or extracellular matrix proteins) influences cell metabolism, drug delivery and distribution. In addition, the existence of a rich tumour microenvironment (TME), influencing cancer cell functions and favouring chemoresistance, has been recently claimed to be an essential factor in cancer stem cell initiation and promotion[9].

PDAC RESISTANCE TO THERAPIES

On account of PDAC aggressive nature and its resistance to therapies, no successful treatment has been introduced so far[10]. In fact, until recently the gold standard in PDAC treatment was gemcitabine. Applied as a first line therapy drug since 1997, gemcitabine modestly improved patients' perspectives, increasing overall survival (OS) for 6 mo compared to previously used fluorouracil (5-FU)[11]. Since that time, attempts have been made to increase the efficacy of PDAC treatment and prolong patient survival; however, only modest or statistically insignificant improvements have been achieved so far. In the last years, two new drug regimens, ABRAXANE and FOLFIRINOX have been introduced[12,13]. However, their application did not increase OS to a meaningful degree when compared to gemcitabine, at the same time escalating the frequency of adverse events. Nevertheless, both treatments have obtained FDA approval and currently ABRAXANE combined with gemcitabine is acknowledged as a standard first-line therapy for pancreatic cancer. Considering the high number of genes altered during PDAC progression, targeted therapies emerged as a potential therapeutic tool. Many small inhibitors have been developed as single agents or applied in combination with gemcitabine or ABRAXANE to enhance their efficacy[14-18]. However, the vast majority of them failed to improve patients' survival in the clinical settings. Therefore, it remains pivotal to gain better knowledge on the mechanisms of PDAC chemoresistance and to

find novel therapeutic strategies in order to develop more effective treatment regimens.

Among other factors, the failure of PDAC treatment has been attributed to local recurrence and liver metastasis and importantly, to its high chemoresistance, both intrinsic and acquired. The phenomenon called multi-drug resistance (MDR), which is characterized by resistance to a broad spectrum of structurally diversified compounds, has been confirmed as one of the main reasons for the inefficiency of PDAC therapies, leading to tragic health and economic consequences.

There are multiple factors contributing to the development of MDR in pancreatic cancer, such as decreased drug uptake, accelerated drug metabolism and DNA repair, blocking of apoptotic pathways, metabolic changes and the presence of highly resistant stem-like cells. Also, high heterogeneity of the tumour, dense stroma and hypoxia impairing drug delivery and constitutive activation of several signalling pathways, including K-Ras, PI3K/Akt, Notch or NF-κB, with the latter being additionally enhanced during chemo- and radiotherapy, all confer the modest response of PDAC to applied therapies[19-23]. Moreover EMT, frequently observed in PDAC tumours, has been implicated in conferring its resistance. Also, acquired mutations in targeted genes and reactivation of parallel pathways add to the therapy failing. However, in most cases the interplay between several of these processes is essential for chemoresistance development[24]. Additionally, high expression of transmembrane proteins belonging to the ATP-binding cassette (ABC) transporter family in tumour specimens is one of the major factors contributing to increased drug efflux and has been connected with MDR, adding to the poor response of PDAC to treatments[25-28]. Apart from drug extrusion, as integral membrane constituents, ABC transporters normally regulate the distribution of a wide variety of molecules, influencing different pathways and biological processes, which suggests their more direct impact on cell physiology and possibly, carcinogenesis. Therefore, the understanding of the role of ABC transporters both in healthy physiology and in cancer is crucial for the development of specific, potent and safe inhibitors that might be used in PDAC therapy.

ABC TRANSPORTERS AS MULTI-DRUG RESISTANCE MECHANISM

One of the main obstacles in cancer therapy is the resistance, both constitutive and acquired to administered drugs. As aforementioned, one of the processes responsible for drug resistance is the decreased intracellular accumulation of the drugs caused by their efflux from the cells induced by the expression of membrane drug transporters belonging to the ABC family.

The family of ABC transporters is a highly conserved family of proteins, expressed in all organisms, which

implies their relevance in many biological functions. To date, 48 human genes and one pseudogene encoding the members of ABC family have been described and grouped into 7 subfamilies (ABCA-G), based on their sequence and structural similarity[29,30]. ABC transporters are integral transmembrane proteins which, by utilizing energy obtained from ATP hydrolysis, which drives the progressive conformational changes in their domains, shuffle molecules across the plasma and intracellular membranes against their gradient[31,32] (Figure 1). The structure of ABC transporters is highly conserved and consists of two hydrophobic transmembrane domains (TMDs), which form a pore in the membrane creating substrate-binding environment linked to two hydrophilic nucleotide-binding domains (NBDs) localized in the cytosol[33,34]. ABC transporters are reported to export a wide variety of structurally diverse endogenous ligands including amino acids, peptides, vitamins, sugars, hormones, ions, lipids and xenobiotics[26,32,35-37]. For example, ABCB1 has been reported to be able to transport more than 200 structurally diversified molecules[38-41]. Additionally, ABC transporters are known to excrete toxins form kidneys, gastrointestinal tract and liver, demonstrating a protective role in those tissues[42]. Few ABC transporters, *e.g.,* ABCC7- cystic fibrosis transmembrane conductance regulator (CFTR) or ABCC8- the sulphonyl urea receptor (SUR1), are not directly involved in transport of molecules across the membrane but use the ATP hydrolysis to regulate the activity of Cl$^-$ and K$^+$ channels respectively[43]. In healthy physiology, ABC transporters are expressed in a wide variety of tissues, mainly associated with biological barriers (Table 1). As an example, ABCC1 is expressed in kidneys, intestine, ovaries, adrenal glands, colon, stomach, testes, lungs and blood-brain barrier and ABCB1 is mostly expressed in gastrointestinal tract, pancreas, kidneys, brain and adrenal glands, where they are involved in diverse physiological functions and in excreting toxins from the cells[40,44,45]. However, their enhanced levels have been found in different cancer types, suggesting the relevance of ABC transporters in cancer and its chemoresistance. So far, 15 of the transporters have been attributed the role of drug pumps, contributing to MDR *in vitro*[46]. Especially, P glycoprotein (P-gp)/ABCB1, breast cancer resistance protein (BCRP)/ABCG2, multidrug resistance protein 1 (MRP1)/ABCC1 and other members of ABCC subfamily (*e.g.,* ABCC2, ABCC3) have been reported to be responsible for PDAC chemoresistance[47].

Up to date, most research has been focused on P-gp, a member of the ABCB subfamily of transporters[48,49]. It exports a wide variety of molecules of "amphipathic nature" including anthracyclines, HIV-protease inhibitors, calcium channel blockers, steroid hormones, antibiotics, lipids, taxanes and alkaloids[50,51]. P-gp overexpression has been observed in several cancers including ovarian, colon, kidney or adrenocortical cancer, correlating with poor prognosis[52,53]. Additionally,

Table 1 Selected ATP-binding cassette transporters, their normal physiological expression and overexpression in cancer tissues[117,171]

ABC transporter	Tissue expression	Cancer overexpression	Correlation with PDAC survival (5-yr survival)
ABCA			
ABCA1	Lung, colon, liver, brain, testicles	Glioma, lung, testis, liver, colorectal, breast, renal cancer,	H: 21%
			L: 29%
ABCA7	Bone marrow, brain, kidney, colon, lung pancreas	Melanoma, Lung, cervical, stomach, endometrial, colorectal, pancreatic cancer	H: 38%
			L: 0%
ABCB			
ABCB1	Brain, blood-brain barrier, colon, liver, kidney, testis, placenta, small intestine, pancreas	Ovarian, breast, colon, kidney, adrenocortical cancer, AML	H: 34%
			L: 20%
ABCB4	Liver	Liver, lung, renal cancer, melanoma	H: 49%
			L: 22%
ABCC			
ABCC1	Kidney, colon, pancreas, lymph nodes, liver, testis, brain, blood-brain barrier, breasts, spleen,	Breast, lung, ovarian or prostate cancer, neuroblastoma	H: 13%
			L: 43%
ABCC2	Brain, lymph nodes, liver, colon, kidney, lung, testis, breasts, pancreas	Colorectal, liver, lung, gastric cancer	H: 29%
			L: 27%
ABCC3	Pancreas, liver, lymph nodes, lung, adrenal glands, colon, testis, spleen, small intestine	Pancreatic, liver, lung, colorectal, stomach, renal, breast cancer	H: 13%
			L: 41%
ABCC4	Brain, testis, colon, kidney adrenal glands, pancreas, liver, ovary, lung, spleen, breasts, skin, heart	Prostate, renal, lung, breast, ovarian, stomach cancer	H: 32%
			L: 23%
ABCC5	Lymph nodes, pancreas, kidney, testis, brain, colon, liver, heart, muscles	Lung, urothelial, breast, cervical, renal cancer, glioma	H: 34%
			L: 0%
ABCG			
ABCG1	Pancreas, liver, colon, kidney, brain, lung, lymph nodes, testis	Lung, renal, breast, endometrial, prostate, colorectal, cervical, pancreatic cancer, glioma	H: 34%
			L: 0%
ABCG2	Intestine, testis, colon, placenta, liver, kidney, small intestine	Liver, testis, prostate, renal cancer, glioma	H: 32%
			L: 23%
ABCG4	Brain, endocrine, testis, colon, liver, kidney	Glioma, melanoma, thyroid, head and neck, renal, testis, ovarian, endometrial cancer	H:43%
			L: 23%

The correlation between the overexpression of the transporters in PDAC and observed 5-year survival is also demonstrated[117]. H: High expression of the transporter; L: Low expression of the transporter. Statistically significant association is highlighted in bold. AML: Acute myeloid leukaemia; PDAC: Pancreatic ductal adenocarcinoma; ABC: ATP-binding cassette.
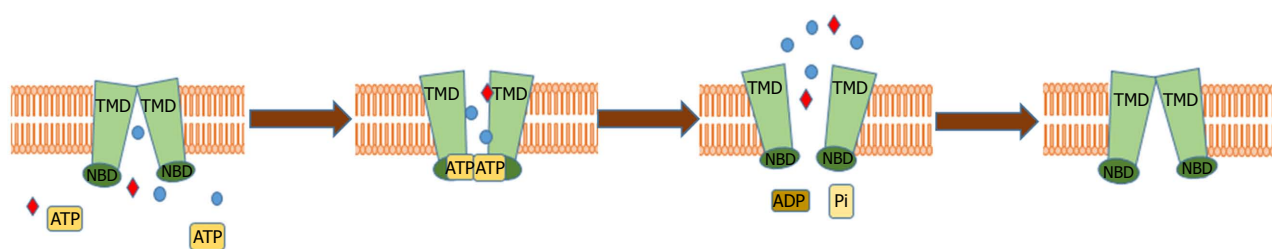

Figure 1 The schematic presentation of the mechanism of ATP-mediated ATP-binding cassette transporter substrate translocation. TMD: Transmembrane domain.

treatment-induced increase in ABCB1 expression has been noted in acute myeloid leukaemia (AML)[54], breast and high-grade bladder cancer[55]. ABCB1 is known to be responsible for developing drug resistance to neutral and cationic hydrophobic compounds, *e.g.*, to anthracyclines (daunorubicin, doxorubicin), colchicines, taxanes (paclitaxel, docetaxel), vinca alkaloids (*e.g.*, vincristine, vinblastine) and tyrosine kinase inhibitors (imatinib)[56-58].

The main role in xenobiotic transport and drug resistance in many cancers has been attributed to the ABCC subfamily of transmembrane transporters[59], with 9 out of 12 members being involved in MDR[47,59]. The most studied of MDR proteins, ABCC1 (MPR1)

has been demonstrated to be expressed in several cancers, including breast, lung, ovarian and prostate cancer, showing the correlation between the expression of ABCC1 and poor patients' outcome[60]. It has been suggested that ABCC1 expression may confer resistance to methotrexate, vinca alkaloids, anthracyclines and camptothecins[47,61,62], influencing drug resistance in plethora of cancers. Additionally, cyclic nucleotides and their analogues (*e.g.,* gemcitabine) may be transported by ABCC4 and ABCC5[62-64], potentially contributing to their ineffectiveness in PDAC therapy.

Resistance to doxorubicin, mitoxantrone, anthracyclines and topotecan (quinolone topoisomerase inhibitor)[65] has been attributed to ABCG2 transporter[66,67], which

functions mainly in the ovaries, brain, liver, prostate, placenta and small intestine[68]. Additionally, increased ABCG2 expression has been reported in pluripotent stem cells, suggesting its role in the maintenance and protection of stem cells[69].

Regardless of the remarkable increase in the knowledge on the ABC transporters structure and MDR induction achieved in the past few decades, the actual function and significance of these proteins is highly underexplored. It is known that in healthy physiology, ABC transporters are involved in drug absorption, distribution and elimination, determining bioavailability of administered drugs. Both apical and basolateral membranes of gastrointestinal tract and biological barriers, in which ABC transporter expression has been demonstrated, need to be penetrated by the drug to reach its target. Therefore, ABC transporters expression may influence pharmacokinetic characteristics of administered chemotherapeutics. Additionally, various other physiological roles have been assigned to ABC transporters such as export of fatty acids, cholesterol, peptides, sterols and xenobiotics. Many ABC transporters are involved in secretion of bioactive molecules and in the transport of signalling lipids, which contribution to cancer progression has been well established. As an example, ABCA1 is involved in reverse cholesterol transport as well as phospholipids transport to plasma membrane[70,71]. Interestingly, recent studies demonstrated ABCC1 as an active player in progression of ovarian and prostate cancer[72,73], by extrusion of lipids (lysophosphatidylinositol, sphingosine 1-phosphate) that have been previously attributed a crucial role in carcinogenesis[73,74]. The changes in cancer cell proliferation, migration, invasion and resistance to apoptosis mediated by the activity of ABC transporters have been also widely documented[75]. Considering that information, attention has been brought to the pivotal role played by ABC transporters in carcinogenesis beyond chemoresistance and to the correlation between their expression with cancer progression and aggressiveness. Nevertheless, this area is still overlooked and more studies need to be focused on this aspect of ABC transporters' activity in order to fully elucidate their role in cancer.

ABC TRANSPORTERS- DRIVERS OF PDAC PROGRESSION?

There have been very limited studies on the role and expression of ABC transporters in pancreatic cancer; however, strong correlation between few of their members and PDAC has been recently suggested. On the basis of mRNA analysis, the expression of ABCC1, ABCC3, ABCC4, ABCC5 and ABCG2 in both pancreatic cancer samples and in healthy pancreas has been demonstrated[76,77] and was correlated with cell resistance to commonly applied chemotherapeutics[78]. At the same time, ABCC6, ABCC8 and ABCC9 could not

be detected in any of the studied pancreatic cancer cell lines[79]. Furthermore, more in depth analysis showed that although ABCG2, ABCC1 and ABCC4 levels did not differ significantly between tumour and healthy tissues, ABCC3 and ABCC5 were found to be remarkably overexpressed in PDAC specimens. Moreover, although expression of none of them could be coupled with cancer stage, the differentiation status and tumour grading were related with increased ABCC3 levels and correlated with poor survival, whereas no such correlation could be found for ABCC5.

ABCC3 transporter is involved in transporting of bile salts and organic ions[80,81]. It has been also implicated in mediation of drug resistance, *e.g.,* to vincristine, methotrexate or etoposide; compounds used in clinical studies for PDAC treatments, which demonstrated only marginal effects[82]. Moreover, its expression levels have been correlated with survival of patients after resection, suggesting possible predictive aspect of ABCC3 expression in PDAC.

ABCC5 is involved in transport of nucleotide analogues; therefore, it is tempting to speculate its involvement in excessive efflux of nucleotide analogues-based drugs, such as 5-FU or gemcitabine. In fact, although still controversial, it has been shown that ABCC5 is responsible for gemcitabine resistance in pancreatic cancer[64,79,83]. Analysis of PDAC specimens demonstrated overexpression of ABCC5 transporter in samples resistant to gemcitabine, suggesting its involvement in the decreased efficiency of the drug. Furthermore, exposure of different PDAC cell lines to gemcitabine, as well as 5-FU/gemcitabine combination significantly increased the expression of ABCC5 demonstrating drug induced mechanism of PDAC cell resistance to the treatment[79,84]. Therefore, although not directly associated with PDAC progression, the importance of ABCC5 in PDAC chemoresistance, both inherent and acquired, makes it a valuable drug target for the enhancement of the efficacy of applied therapies.

While the role of ABC transporters in mediating chemoresistance is well established, little is known about their direct, drug-efflux independent contribution to pancreatic cancer progression. Nevertheless, intensive studies in recent years suggest that beyond their role in drug resistance, the biological functions of ABC transporters are more complex. It has been proposed that tumour-promoting functions of ABC transporters are based on their ability to export active signalling molecules and hormones, which by autocrine or paracrine regulation activate cancer cells as well as tumour environment. Increasing interest in this area has demonstrated the significant impact of these proteins on invasion, migration and differentiation of malignant cells[75]. Also, changes in metabolism as well as redox status, characteristics pivotal in PDAC tumorigenesis, may be induced by ABC transporters-released molecules.

One of the major events in PDAC development

is the metabolic switch, which occurs in response to decreased nutrient and oxygen supply[85-87]. Increased glucose dependence and use or aerobic glycolysis for energy production, known as Warburg effect, allows quickly proliferating PDAC cells to survive under harsh conditions and is considered as one of the hallmarks of cancer[88]. Additionally, glutamine dependence and increased protein breakdown add to cancer cell high proliferative abilities. However, a small population of cells with stem-like characteristics, which reside the areas of the tumour lacking oxygen and glucose supply, are known to rely on mitochondrial oxidative phosphorylation rather than glycolysis, which results in increased ATP production. This phenomenon may add to increased activity of ABC transporters observed in cancer cells. Therefore, low oxygen and nutrient supply may contribute to PDAC resistance by increase of the ABC transporters levels and their ATP-dependent substrate transport, suggesting a possible mechanism of hypoxia-induced chemoresistance, tumour maintenance and cancer progression.

Apart from glucose and glutamine addiction, increased lipid metabolism and demand has been recently demonstrated for PDAC[89,90]. Bioactive phospholipids are directly involved in the induction of cancer cell proliferation and thereby, cancer progression[91]. Increase in the levels of saturated lipids helps cancer cells to acquire additional resistance to oxidative stress by consolidating the membranes. Both, de novo lipid synthesis and their increased uptake have been reported in PDAC[92,93]. Moreover, enzymes involved in lipolysis and lipogenesis are overexpressed in PDAC and are usually correlated with poor prognosis[90]. It has been demonstrated by our work that, in prostate and ovarian cancer, ABCC1-transported lysophosphatidylinositol activates GPR55 receptor forming an autocrine loop, which activation triggers signalling cascade inducing cell proliferation[72]. Phospholipids transport has been also reported for another member of ABC transporter family, ABCG1. Therefore, it is tempting to suspect the existence of a similar mechanism, involving ABC transporter-mediated phospholipid activation of cancer cells in PDAC. An essential factor in PDAC cell survival is also cholesterol availability. As a component of lipid rafts, it influences membrane composition and integrity and interacts with membrane-bound proteins, facilitating activation of phosphorylation cascades[90]. The essential role played by cholesterol in PDAC tumorigenesis limits the growth and division of PDAC cells, depending on its availability[94]. A recent study by Mohelnikova-Duchonova *et al*[95] showed an upregulation in transcript levels of several ABC transporters in PDAC compared to non-neoplastic tissues. Particularly, upregulation of 2 members of ABCA family, ABCA1 and ABCA7 involved in cholesterol export, together with expression of ABCG1 transporting phosphatidylserine, phosphatidylcholine and sphingomyelin, suggests their involvement in cellular cholesterol imbalance in the disease[95]. Another

of the characteristics of PDAC is the highly inflammatory environment, which actively promotes cancer cell proliferation and survival, angiogenesis and assists the metastatic spread[96]. Chronic inflammation, that aids the tumorigenesis and at the same time is one of the main factors contributing to its initiation, is mediated by prostaglandin-mediated pathways. Therefore, the main inflammatory molecules- prostaglandins and leukotrienes are considered as significant players in PDAC development. The prostaglandin-mediated PDAC progression may involve activation of PI3K-Akt signalling pathway, a major player in PDAC progression, increase in VEGFA expression and stimulation of angiogenesis and support of the inflammatory environment[97]. It is now known that several ABC transporters, mainly belonging to the ABCC subfamily (ABCC1, ABCC2, ABCC4) are involved in prostaglandins efflux outside of the cells, enabling the activation of the G protein-coupled receptors, triggering cancer progression[75,98]. Therefore, the manipulation of ABC transporter activity blocking prostaglandin signalling represents an additional potential therapeutic tool. Additionally, due to the proved contribution of leukotriene C4 (LTC4) to PDAC progression[99], its induced pathways have been widely studied as potential drug targets. Regarding the involvement of ABC transporters in leukotriene release, their inhibition presents an additional possibility for LCT4-signalling blockade, influencing cancer development.

Elevated levels of reactive oxygen species (ROS), inducing oxidative stress are also implicated in PDAC initiation and progression[100]. One of the molecules responsible for the maintenance of redox status in homeostasis is glutathione (GSH)[101], which transport is activated in response to oxidative stress. It is also involved in several signalling processes regulating cell proliferation, apoptosis or immune response. Several members of ABCC family (ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC7) and ABCG2 mediate glutathione transport, suggesting their involvement in cellular response to the oxidative stress. Also, ABCB10 has been implicated in cellular protection from oxidative stress[102]. Moreover, oxidative stress induces the activation of NF-κB and Nrf2 signalling, which in turn enhances expression of ABCB1, ABCG2 and ABCC2, additionally contributing to cancer cell resistance[103,104]. Therefore, manipulation of the activity of ABC transporters in cancer cells might potentially increase their antioxidant capacity, which has been shown to provide additional anti-tumorigenic protection[105].

Additionally, tumour environment and its engagement in cancer progression and metastatic spread has emerged as key player in carcinogenesis. Considering the significant role of stroma in PDAC progression and in the development of tumour chemoresistance, targeting its components presents a tempting approach in the development of novel therapies. However, the attempts to deplete stroma have not provided satisfactory results so far. The most promising combination of gemcitabine

and Hedgehog inhibitor IPI-926-03 tested by Olive *et al*[106] has failed due to high toxicity and lack of effectiveness in clinical trials[107]. Currently, molecules targeting hyaluronic acid, combined with chemotherapy, are being tested in phase II and III clinical trials[108]. Nevertheless, investigation of new approaches to target stroma in order to increase chemotherapy efficiency, as well as restraining tumour expansion remains essential. Recently, expression of several of ABC transporters in PDAC stroma has been reported. One of the main stromal components- macrophages- have been demonstrated to express several of the drug transporters, inter alia ABCC1 and ABCC3, contributing to both chemoresistance and tumour progression[109]. Therefore, considering the involvement of ABC transporters in chemoresistance and an emerging role in tumorigenesis, therapies targeting ABC transporters might prove to be useful in depleting or reprograming cancer stroma and reversing cancer resistance to applied drugs. Additionally, expression of few of ABC transporters in non-neoplastic tissues has been recently reported to influence PDAC progression and to be predictive of patients' overall response.

Finally, the most aggressive tumours are composed of non-differentiated cells possessing highly proliferative abilities[75], called cancer stem cells (CSCs). In particular, the existence and high importance of CSCs in cancer resistance to chemotherapy and its involvement in disease recurrence has been suggested for PDAC. Interestingly, high expression of ABC transporters has been reported in less differentiated tumour zones, conferring them a more aggressive phenotype[110-112], also in PDAC[76]. Therefore recently, the interest in CSCs as drivers of resistance and aggressive nature has emerged in PDAC[113,114]. A noticeable characteristic of cancer stem cells is the high expression of members of the ABC transporters family compared to more differentiated cells[115]. Also, it is speculated that their expression profile may be considered as the indicator of stem cell formation and carcinogenic potential of the tissue[116]. Considering the association of cell differentiation levels with its proliferative potential, the overexpression of ABC transporters in cancer stem cells highly supports their contribution to the more aggressive nature of the PDAC. Overexpression of ABC transporters in cancer stem cells may assist in their survival by efflux of xenobiotics, exhibiting protective roles, sustaining their proper performance and maintaining self-renewal characteristics. Additionally, their enhanced expression and activity in cancer cells and especially in CSCs, suggests an additional role in maintaining cancer cells aggressive biology and makes them an attractive therapeutical target.

ABC TRANSPORTERS EXPRESSION PROFILES AS PROGNOSTIC MARKERS IN PDAC

Although the investigation on the role of ABC transporters in PDAC is still in its outset, the initial analysis suggests their probable contribution to PDAC development and points at potential beneficial clinical consequences. Database analysis showed that the high importance and the potential of ABC transporters as pharmacological targets in PDAC is reflected in the association of the expression of its individual members with the prognosis of patients' survival[117]. Notable correlation between observed 5-year survival and expression of a majority of ABC transporters has been observed (Table 1); however, this discovered association is not uniform. Significant reduction in survival probability has been attributed to high expression of *e.g.*, ABCA1, ABCA12, ABCB1, ABCC1, ABCC3 or ABCC7. Expression of few other ABC transporters showed similar trend, nonetheless, their relationship with the OS was not remarkably pronounced. On the other hand, higher expression of a substantial number of ABC transporter genes has been correlated with increased chance of PDAC patients' survival. Among others, the expression of ABCA2, ABCA7, ABCB6 ABCB8, ABCC5 or AGCG1 in PDAC tissues most markedly correlated with prolonged 5-year survival, suggesting their-mediated release of molecules of anti-tumorigenic characteristics and favourable prognostic potential.

Considering the elevated expression of multiple ABC transporters in a vast majority of cancers and their redundancy in substrate specificity and activity, determination of their expression profiles and their clustering in prognostic groups, rather than analysis of individual members, also raised a lot of interest in the last years. The existence of ABC transporters expression signatures in PDAC and their correlation with clinic-pathological characteristics of the tumours has been studied by Mohelnikova-Duchonova *et al*[95], and dysregulation of expression of several members of ABC family has been observed. Upregulation of ABCB4, ABCB11, ABCC1, ABCC3, ABCC5, ABCC10 and ABCG2 has been noted in PDAC, compared to non-neoplastic tissues. Surprisingly however, expression of few ABC transporters in non-neoplastic tissues also could be correlated with tumour progression and survival. Moreover, higher levels of T3 and T4 stages were associated with ABCA1 and ABCB3 upregulation and ABCG1 and ABCG2 downregulation. In contrast, smaller size tumours were connected with the cluster, in which ABCA8, ABCB5, ABCA9, ABCA10 and ABCC9 were upregulated, while downregulation of ABCA12, ABCA13, ABCC3, ABCC7 and ABCC13 has been noted. Similarly, ABCB9 and ABCC4 upregulation correlated with N1 status, while ABCA3, ABCD1 overexpression and ABCA6 and ABCC10 downregulation corresponded with increased angioinvasion.

This and previous studies demonstrated the correlation of ABC transporter expression in tumour specimens with clinic-pathological features in different cancer types[118]. Nevertheless, the high importance of tumour microenvironment and its proposed involvement in PDAC progression, suggests that ABC transporter

Table 2 Selected ATP-binding cassette transporters responsible for the development of multi-drug resistance, their experimental inhibitors and drug specificity

ABC transporter	Inhibitor	MDR	Ref.
ABCB1	I generation:Cyclosporine A, Verapamil II generation: Valspodar, zosuquidar III generation: Tariquidar, OC144-093	Daunorubicin, epirobicin, doxorubicin, colchicines, paclitaxel, docetaxel, vincristine, vinblastine, imatinib	[46,145,146,171]
ABCC1	MK571, probenecid, ibrutinib, 3ATA	Anthracyclines, vinca alkaloids, camptothecins, daunorubicin, imatinib, etoposide, vincristine, vinblastine, methotrexate	[46,145,146,172]
ABCC2	Metothrexate, cyclosporine A, fluorescein, MK571	Doxorubicin, cisplatin, irinotecan, epirubicin, vinblastine	[46,145,146,171,173]
ABCC3	Indomethacin, sufinpyrazone, probenecid, benzmromarone	Etoposide, methotrexate, teniposide	[46,145,146,171,173]
ABCC5	Curcumin, trequensin, sildenafil	Gemcitabine, methotrexate, 6-mercaptopurine	[46,145,146,171,173]
ABCG2	Fumitremorgin C, Ko143, GF120918	Daunorubicin, doxorubicin, irinotecan, mitoxantrone, methotrexate, epirubicin, etoposide	[46,145,146,171]

MDR: Multi-drug resistance; ABC: ATP-binding cassette.

expression in non-neoplastic tissues might have important clinical implications. Following the analysis of 27 non-neoplastic pancreatic tissues and pairing them with 32 PDAC samples, 4 different clusters could be distinguished based on the gene expression profiles in cancer *vs* normal specimens. PN1 and PN2 clusters were characterized by upregulation of the majority of ABC transporters genes and correlated with significantly shorter patients' overall survival (OS) than patients grouped into PN3 and PN4 clusters, in which significant downregulation of genes or heterogeneous gene expression has been observed[119]. Especially, ABCA2, ABCA4, ABCA5, ABCC2 and ABCD4 signatures showed significant difference in patients' survival when comparison between upregulated and downregulated genes was carried out. Additionally, tumour-node-metastasis, age, gender, disease stage, margin status, therapy and survival have been analysed; however, no significant correlation between those features and ABC profiles could be established. Although the study presented few limitations, such as small group size or the distance between collected tumours and control tissue, created expression clusters could be successfully implemented into clinical practice. Moreover, reduction of the analysed genes to the limited group showing most distinct expression, did not have any impact on the statistical significance of observed clinic-pathological correlations, creating more practical and convenient clinical prognostic tools.

ABC TRANSPORTERS IN CANCER THERAPY

Looking at the key role played by ABC transporters in cancer chemoresistance and the emerging knowledge on their crucial contribution to tumorigenesis, the development of targeted therapies, aiming to block or modulate their activity has become a crucial area in cancer research. Inhibition of transporter activity, arrest

of the transcription factors regulating their expression or blockade of the transporter-induced signalling pathways represent the options for impeding ABC transporters activity[120]. So far, 3 generations of ABC transporters modulators, directed mainly against ABCB1, have been developed[120,121] (Table 2). The first generation inhibitors, such as verapamil, quinine or cyclosporine A, compounds previously established for other conditions, in spite of promising *in vitro* activity[122,123], showed significant toxicity, unacceptable for further usage[123,124]. Lack of potency and specificity, combined with pharmacokinetic complications restrained their further investigation[125]. Structural modifications of existing inhibitors, aiming to enhance their efficacy and specificity, at the same time decreasing observed adverse effects, also did not provide satisfactory results. Valspodar (cyclosporine A derivative), a second generation ABCB1 inhibitor, demonstrated enhanced efficiency accompanied by decreased toxicity[126]. However, it showed unsatisfactory results in the majority of clinical trials, in which its co-administration with chemotherapeutics, *e.g.*, carboplatin, paclitaxel or doxorubicin did not exhibit any benefits, and in some cases deteriorated patients' outcome[127,128]. Likewise, application of dofequidar or biricodar citrate (VX-710)[129] did not result to be favourable, as their use has been restricted by the potential interactions with anti-cancer therapeutics (vincristine or paclitaxel)[130]. All these limitations led to the development of a third generation of inhibitors which potency, due to the rational QSAR design, has been described as 200-fold higher than the previously developed anti-ABCB1 molecules, greatly enhancing drug accumulation[131]. Additionally, only minimal drug-drug interactions have been reported. Clinical trials have been commenced for zosuquidar (LY335979)[132], elacridar (F12091)[133], mitotane (NSC-38721)[134], annamycin[135] or tarquidar (XR9576)[136]. Nevertheless disappointingly, most of the clinical trials testing their applicability have been

discontinued due to lack of significant positive response and off-site effects.

There are several reasons for the lack of success of the ABC transporters inhibition. Increased toxicity caused by off-target action in healthy tissues, as well as their high doses were the main reasons for the discontinuation of the trials for first and second generation inhibitors[42]. Increasing evidence of substrate similarities between ABC transporters and CYP450, enzyme involved in drug metabolism, suggests interactions of tested compounds with the enzyme, which influences pharmacokinetic properties of co-administrated chemotherapeutics, changing their activity, lowering the efficacy and, as a consequence, increasing the toxicity[137]. Therefore single-agent application of ABC transporters inhibitors should be considered in future research. Another reason for high toxicity of these modulators has been attributed to decreased clearance of anticancer agents and natural xenobiotics caused by unspecific blockade of the transporters. As an example, ABCB1 inhibition, apart from cancer cells may also result in its blockade in canalicular membrane in healthy liver or kidney, reducing the clearance of chemotherapeutics[42,138]. The involvement of some of the ABC transporters (mostly ABCB and ABCC subfamilies) in the immune system is another obstacle, as disruption of its proper functioning may result in undesirable deterioration in anti-cancer immune responses[139]. The ineffectiveness of targeted therapies may also lay in the functional redundancy of several ABC transporters, highly impairing full efficiency of the blockade of individual protein. Another limitation in the presented approach has been the fact that the vast majority of studies have been focused on ABCB1. Nevertheless, with increasing evidence of the role of other ABC transporters in cancer, the inhibitors of ABCC1 (*e.g.,* probenecid, sulindac, biricodar, BAY-u9773 or MK571)[129,140-142], ABCG2 (Ko143, fumitremorgin C, genistein, biochanin A)[143,144] or ABCC3 (indomethacin or sulfinpyrazone)[145,146] have been considered (Table 2). However, some of them similarly to ABCB1 blockers, exhibited unfavourable toxicity levels when combined with chemotherapy. Additionally, several non-selective ABCB1 inhibitors have been tested for their activity towards other ABC transporters[146]. Nonetheless, as the interest in ABC transporters increased only recently, the efficacy of the abovementioned therapeutic approach still needs to be evaluated. Also, the majority of the studies conducted so far have been focused on the reversal of chemoresistance rather than influencing cancer progression. However, current knowledge on the additional, or maybe principal role of ABC transporters in tumorigenesis might shed more light on the basis of current inhibitors toxicity as well as could allow for exploration of novel more specific molecules, aiming at slowing down cancer progression, rather than reversing MDR.

Considering the marginal effectiveness of ABC transporters inhibitors achieved so far, alternative concepts for ABC transporters targeting are being tested (Figure 2).

RNA interference, use of monoclonal antibodies, antisense oligonucleotides or the use of transcription regulators is currently under consideration[147-150]. miRNA use has been also claimed as a possible way for ABC transporter regulation and reversal of chemoresistance[151,152]. As crucial players in carcinogenesis, also confirmed in PDAC, miRNA regulation has been proposed as an interesting therapeutic tool[153]. To date, several miRNAs have been reported to inhibit the expression of different ABC transporters, having chemotherapeutic effects[154-156]. Moreover, it has been demonstrated that tyrosine kinase inhibitors may block ABC transporters by binding to their transmembrane domain at substrate-binding sites[157]. Imatinib, nilotinib, sunitinib or lapatinib, drugs tested for the PDAC therapy independently of their ABC-inhibiting properties, have been demonstrated to block ABCB1, ABCC2 or ABCC10[158-161]. However, this approach also needs further evaluation.

Currently, the use of nanoparticles for the delivery of therapeutics to the target cells has emerged as a growing area of interest[162,163]. Their small size, together with increased surface area, enhances the stability and solubility of the administered drugs, improving their bioavailability[164]. Additionally, controlled, prolonged release and protection form degradation present further advantages of that therapeutic approach. Co-delivery of the inhibitors of ABC transporters and chemotherapeutics with the use of nanoparticles is also applied to minimize observed side effects occurring as a result of drug-drug interactions. Nevertheless, the emerging field of the manipulation of ABC transporter activity for therapeutic purposes is still in its outset and more studies are needed to fully assess their pharmacological potential.

DISCUSSION

In the last years, ABC transporters have attracted remarkable attention of researchers from different scientific areas. The role of ABC transporters in different physiological and pathological conditions, including cancer, has been widely reported, increasing the interest in the development of their specific inhibitors. Especially, the well-known involvement of ABC transporters in the development of multi-drug resistance (MDR) led to the investigation of the potential of its reversal by blocking ABC transporter activity. Clinical relevance of several ABC transporters in multi-drug resistance reversal has been primarily attributed to P-gp, ABCG2, ABCB4 and 4 members of ABCC subfamily- ABCC1, ABCC2, ABCC3 and ABCC4[165]. Therefore, the main focus so far has been placed on these proteins in terms of their pharmacological potential. However, in spite of the initial enthusiasm regarding ABCB1 inhibitors, their efficacy in clinical settings has failed to provide any improvements, leading to the early closure of the trials[166,167]. Considerably high toxicity caused by lack of specificity and changes in pharmacokinetic of co-applied chemotherapeutics, decreasing their efficacy were
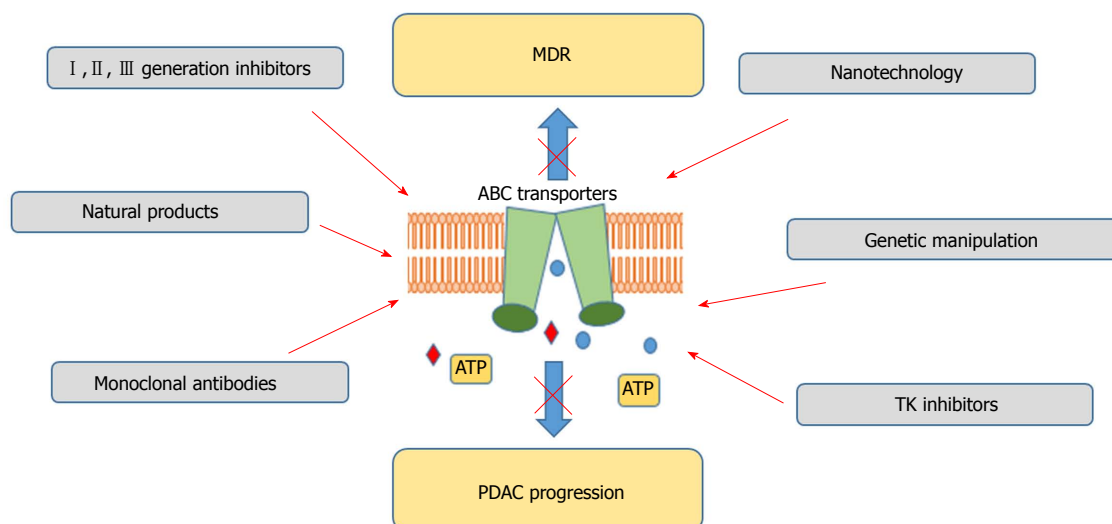
Figure 2 Pharmacological approaches towards inhibition of ATP-binding cassette transporters. MDR: Multi-drug resistance; PDAC: Pancreatic ductal adenocarcinoma; ABC: ATP-binding cassette; TK: Tyrosine kinase.

some of the reasons for the disappointing results[168]. The successful implementation of developed inhibitors was strongly impeded by the complexity of ABC transporters functioning. The correlation between cancer chemoresistance and ABC transporters expression is two-sided and forms a specific loop, which may increase cancer resistance to applied therapies. On one hand, their expression contributes to enhanced drug efflux from the cells, diminishing their efficacy, on the other hand, many studies have reported increased expression of the transporters, induced by drugs application, complementing formed loop. Therefore, in spite of the enhancement of drug accumulation and reversal of induced chemoresistance demonstrated *in vitro*, little success has been reported during clinical trials. Also, increased toxicity and insufficient potency observed during clinical trials restrained the majority of tested compounds from the clinical use. Additionally, the majority of carried clinical trials were performed on patients previously treated with several anticancer therapeutics. Therefore, the assessment of the protein levels might have been misevaluated due to drug-induced enhancement of expression of ABC transporters. Moreover, several of the studies were designed without proper patient stratification for ABC transporters expression. As an example, little success rate in ovarian cancer patients, might be explained by low expression rate of P-gp in this tumour type[127]. Although reversal of the drug resistance was the principal goal of ABC-targeted therapies, considering the increasing awareness of the pivotal role of ABC transporters beyond chemoresistance, their specific inhibition might not only aid to increase the activity of other therapeutics, but directly balk tumour development and progression[56], encouraging their further exploration. Therefore, the repertoire of ABC transporters against which inhibitors are being developed should be expanded for those playing an active role, not only in MDR, but in the

expulsion of bioactive molecules. Looking at the wide variety of substrates transported by ABC transporters, together with their increased expression in cancer cells and especially cancer stem cells, the role of these proteins in the transport of signalling molecules, which activity promotes cancer progression, has become an area of interest. High impact of bioactive lipids, including phospholipids, sphingolipids or cholesterol on PDAC tumorigenesis and an emerging role of ABC transporters in their release presents a novel opportunity for targeting the disease. It has been previously demonstrated that one of the hallmarks of PDAC is lipid-dependence and that the decrease of the lipids levels may reduce cancer progression. Accordingly, aiming to block specific ABC transporters responsible for their extrusion, mainly members of ABCA and ABCC subfamilies, and depriving cancer cells of the necessary fuel may highly contribute to slowing down PDAC development. In fact, it has been demonstrated in several cancers that targeting of ABC transporters involved in lipid transport (*e.g.*, ABCC1 in prostate or ovarian cancer or ABCC4 in neuroblastoma) showed significant improvement in *in vitro* and *in vivo* models[75], slowing down cancer progression. Therefore, single-agent therapies based on ABC transporter inhibition should be considered to target cancer progression. Moreover, patients' treatment with ABC transporters single inhibitors would eliminate the risk of drug-drug interactions, reducing the risk of adverse events.

Importantly, the expression of ABC transporters may not only be explored in terms of their pro-tumorigenic activity but may also serve as prediction of therapy efficiency and patients' outcome. Database analysis demonstrated strong influence of the expression of the transporters *e.g.,* ABCC3 or ABCC1 on reported 5-year survival. However, positive association of other transporters (*e.g.,* ABCC5 or ABCA7) with the increased survival demonstrates the complexity of the role of ABC

transporters in PDAC tumorigenesis. It also shows the necessity for enhanced research in this area to fully understand and explore the therapeutic potential of these transmembrane proteins in PDAC therapy. The enhancement of chemotherapy efficacy, *e.g.,* by ABCC5 blocking, has been demonstrated for the gemcitabine-based therapies. However, considering the favourable association of this transporter with PDAC patients' survival, it is tempting to speculate that its inhibition might interfere with some of the protective functions that ABCC5 might exhibit and, as a consequence, deteriorate patients' outcome. Also, despite being overexpressed in a majority of cancer types, the role of ABC transporters is not uniform. Negative or positive correlation of the protein expression and survival observed in PDAC patients, is not invariably reflected in other cancer types. As an example ABCA7, expressed at a similar level in pancreatic and lung cancer, although positively correlated with 5-year survival in the PDAC (38% high expression *vs* 0% low expression), has no statistically significant effect in the latter case (48% *vs* 43%)[117]. Therefore, studying the context accompanying ABC transporters expression and functioning is of high importance in order to stratify their individual members in context of their pharmacological potential in diverse cancers. Additionally, the focus of research should not be placed only on the potential of the inhibition of ABC transporters that have undermining roles in carcinogenesis. Hence, the investigation of the characteristics of the ABC transporters that favour the survival of PDAC patients should be also explored to study the mechanisms and molecules responsible for their protective function.

Finally, ABC transporters profiling in cancer has proven to provide a potent tool in estimation of patients' response to applied therapies. As an example, analysis of 21 breast cancer specimens before and after neoadjuvant treatment showed different expression of several ABC transporters[169]. Similarly, 6 ABC transporters genes in AML samples allowed for their organization in two expression groups, correlated with resistance and patients' prognosis[170]. Correspondingly, generation of ABC transporter expression profiles in PDAC has allowed for creation of clusters, characterized by differentiated expression of their individual members. Correlation of each cluster with a variety of disease parameters (*e.g.,* number of metastases or drug response) and more importantly, with patients' survival suggested the gene profiling for ABC transporters expression as a clinically relevant prognostic tool.

CONCLUSION

Although a lot of advancement has been achieved in the identification of new druggable targets involved in PDAC progression and chemoresistance, no significant improvement in transferring that knowledge into clinical practice has been accomplished, leaving PDAC

patients with grim prognosis. As critical players in PDAC chemoresistance and disease development, ABC transporters seem a promising target for the development of novel targeted therapies. However, despite their remarkable pharmacological potential demonstrated *in vitro*, acquired knowledge has not been successfully implemented in the clinic yet. Nevertheless, the knowledge learnt from previous mistakes and the potential reasons for the failed implementation of the inhibitors should be considered in the development of new studies and treatments. In the light of recent data, the potential of few ABC transporters beyond MDR reversal should be further explored to fully scrutinize the applicability of ABC transporter inhibition for clinical practice. More emphasis on the ABC transporters involvement in PDAC progression should be placed in prospective studies, leading to the determination of the proteins with the most pharmacological potential followed by design of single-agent treatment. The knowledge on the involvement of ABC transporters in cancer metabolic swift, their role in tumour-microenvironment cross-talk should be additionally expanded. Animal models of pancreatic cancer should be implemented in the development of new potential inhibitors to investigate their impact on abovementioned processes. In conclusion, proper study design and patients stratification regarding ABC transporters expression leading to tailored therapies should be elucidated in order to add to the efficiency of administered drugs.

REFERENCES

1   ASCO.org CN. Pancreatic Cancer: Statistics. Available from: URL: https://www.cancer.net/cancer-types/pancreatic-cancer/statistics
2   **Garrido-Laguna I**, Hidalgo M. Pancreatic cancer: from state-of-the-art treatments to promising novel therapies. *Nat Rev Clin Oncol* 2015; **12**: 319-334 [PMID: 25824606 DOI: 10.1038/nrclinonc.2015.53]
3   **Vaccaro V**, Sperduti I, Vari S, Bria E, Melisi D, Garufi C, Nuzzo C, Scarpa A, Tortora G, Cognetti F, Reni M, Milella M. Metastatic pancreatic cancer: Is there a light at the end of the tunnel? *World J Gastroenterol* 2015; **21**: 4788-4801 [PMID: 25944992 DOI: 10.3748/wjg.v21.i16.4788]
4   **Maitra A**, Hruban RH. Pancreatic cancer. *Annu Rev Pathol* 2008; **3**: 157-188 [PMID: 18039136 DOI: 10.1146/annurev.pathmechdis.3.121806.154305]
5   **Yachida S**, Iacobuzio-Donahue CA. Evolution and dynamics of pancreatic cancer progression. *Oncogene* 2013; **32**: 5253-5260

[PMID: 23416985 DOI: 10.1038/onc.2013.29]

6   **Abel EV**, Simeone DM. Biology and clinical applications of pancreatic cancer stem cells. *Gastroenterology* 2013; **144**: 1241-1248 [PMID: 23622133 DOI: 10.1053/j.gastro.2013.01.072]

7   **Wang S**, Huang S, Sun YL. Epithelial-Mesenchymal Transition in Pancreatic Cancer: A Review. *Biomed Res Int* 2017; **2017**: 2646148 [PMID: 29379795 DOI: 10.1155/2017/2646148]

8   **Feig C**, Gopinathan A, Neesse A, Chan DS, Cook N, Tuveson DA. The pancreas cancer microenvironment. *Clin Cancer Res* 2012; **18**: 4266-4276 [PMID: 22896693 DOI: 10.1158/1078-0432.ccr-11-3114]

9   **Charles N**, Ozawa T, Squatrito M, Bleau AM, Brennan CW, Hambardzumyan D, Holland EC. Perivascular nitric oxide activates notch signaling and promotes stem-like character in PDGF-induced glioma cells. *Cell Stem Cell* 2010; **6**: 141-152 [PMID: 20144787 DOI: 10.1016/j.stem.2010.01.001]

10   **Adamska A**, Domenichini A, Falasca M. Pancreatic Ductal Adenocarcinoma: Current and Evolving Therapies. *Int J Mol Sci* 2017; **18**: pii: E1338 [PMID: 28640192 DOI: 10.3390/ijms18071338]

11   **Burris HA 3rd**, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* 1997; **15**: 2403-2413 [PMID: 9196156 DOI: 10.1200/JCO.1997.15.6.2403]

12   **Conroy T**, Desseigne F, Ychou M, Bouché O, Guimbaud R, Bécouarn Y, Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardière C, Bennouna J, Bachet JB, Khemissa-Akouz F, Péré-Vergé D, Delbaldo C, Assenat E, Chauffert B, Michel P, Montoto-Grillot C, Ducreux M; Groupe Tumeurs Digestives of Unicancer; PRODIGE Intergroup. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med* 2011; **364**: 1817-1825 [PMID: 21561347 DOI: 10.1056/NEJMoa1011923]

13   **Von Hoff DD**, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, Seay T, Tjulandin SA, Ma WW, Saleh MN, Harris M, Reni M, Dowden S, Laheru D, Bahary N, Ramanathan RK, Tabernero J, Hidalgo M, Goldstein D, Van Cutsem E, Wei X, Iglesias J, Renschler MF. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N Engl J Med* 2013; **369**: 1691-1703 [PMID: 24131140 DOI: 10.1056/NEJMoa1304369]

14   **Moore MJ**, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, Au HJ, Murawa P, Walde D, Wolff RA, Campos D, Lim R, Ding K, Clark G, Voskoglou-Nomikos T, Ptasynski M, Parulekar W; National Cancer Institute of Canada Clinical Trials Group. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* 2007; **25**: 1960-1966 [PMID: 17452677 DOI: 10.1200/jco.2006.07.9525]

15   **Li Y**, Sun J, Jiang Z, Zhang L, Liu G. Gemcitabine and S-1 combination chemotherapy versus gemcitabine alone for locally advanced and metastatic pancreatic cancer: a meta-analysis of randomized controlled trials in Asia. *J Chemother* 2015; **27**: 227-234 [PMID: 25790948 DOI: 10.1179/1973947815y.0000000013]

16   **Kindler HL**, Ioka T, Richel DJ, Bennouna J, Létourneau R, Okusaka T, Funakoshi A, Furuse J, Park YS, Ohkawa S, Springett GM, Wasan HS, Trask PC, Bycott P, Ricart AD, Kim S, Van Cutsem E. Axitinib plus gemcitabine versus placebo plus gemcitabine in patients with advanced pancreatic adenocarcinoma: a double-blind randomised phase 3 study. *Lancet Oncol* 2011; **12**: 256-262 [PMID: 21306953 DOI: 10.1016/s1470-2045(11)70004-3]

17   **Herrmann R**, Bodoky G, Ruhstaller T, Glimelius B, Bajetta E, Schüller J, Saletti P, Bauer J, Figer A, Pestalozzi B, Köhne CH, Mingrone W, Stemmer SM, Tàmas K, Kornek GV, Koeberle D, Cina S, Bernhard J, Dietrich D, Scheithauer W; Swiss Group for Clinical Cancer Research; Central European Cooperative Oncology Group. Gemcitabine plus capecitabine compared with gemcitabine alone in advanced pancreatic cancer: a randomized, multicenter, phase III trial of the Swiss Group for Clinical Cancer Research

and the Central European Cooperative Oncology Group. *J Clin Oncol* 2007; **25**: 2212-2217 [PMID: 17538165 DOI: 10.1200/jco.2006.09.0886]

18   Momenta Pharmaceuticals Inc. M402 in combination with nab-Paclitaxel and gemcitabine in pancreatic cancer. 2017 In: ClinicalTrials.gov [Internet]. Avaliable from: https://clinicaltrials.gov/ct2/show/NCT01621243. Accessed on: 15.05.2017

19   **Trédan O**, Galmarini CM, Patel K, Tannock IF. Drug resistance and the solid tumor microenvironment. *J Natl Cancer Inst* 2007; **99**: 1441-1454 [PMID: 17895480 DOI: 10.1093/jnci/djm135]

20   **DI C**, Zhao Y. Multiple drug resistance due to resistance to stem cells and stem cell treatment progress in cancer (Review). *Exp Ther Med* 2015; **9**: 289-293 [PMID: 25574188 DOI: 10.3892/etm.2014.2141]

21   **Durand RE**, Olive PL. Resistance of tumor cells to chemo- and radiotherapy modulated by the three-dimensional architecture of solid tumors and spheroids. *Methods Cell Biol* 2001; **64**: 211-233 [PMID: 11070841 DOI: 10.1016/S0091-679X(01)64015-9]

22   **Liu YY**, Han TY, Giuliano AE, Cabot MC. Ceramide glycosylation potentiates cellular multidrug resistance. *FASEB J* 2001; **15**: 719-730 [PMID: 11259390 DOI: 10.1096/fj.00-0223com]

23   **Jain RK**. Delivery of molecular and cellular medicine to solid tumors. *Adv Drug Deliv Rev* 2012; **64**: 353-365 [PMID: 24511174 DOI: 10.1016/j.addr.2012.09.011]

24   **Gillet JP**, Gottesman MM. Overcoming multidrug resistance in cancer: 35 years after the discovery of ABCB1. *Drug Resist Updat* 2012; **15**: 2-4 [PMID: 22465109 DOI: 10.1016/j.drup.2012.03.001]

25   **Gottesman MM**, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2002; **2**: 48-58 [PMID: 11902585 DOI: 10.1038/nrc706]

26   **Higgins CF**. ABC transporters: from microorganisms to man. *Annu Rev Cell Biol* 1992; **8**: 67-113 [PMID: 1282354 DOI: 10.1146/annurev.cb.08.110192.000435]

27   **Lage H**. An overview of cancer multidrug resistance: a still unsolved problem. *Cell Mol Life Sci* 2008; **65**: 3145-3167 [PMID: 18581055 DOI: 10.1007/s00018-008-8111-5]

28   **Shukla S**, Ohnuma S, Ambudkar SV. Improving cancer chemotherapy with modulators of ABC drug transporters. *Curr Drug Targets* 2011; **12**: 621-630 [PMID: 21039338 DOI: 10.2174/138945011795378540]

29   **Dean M**. The genetics of ATP-binding cassette transporters. *Methods Enzymol* 2005; **400**: 409-429 [PMID: 16399363 DOI: 10.1016/s0076-6879(05)00024-8]

30   **Vasiliou V**, Vasiliou K, Nebert DW. Human ATP-binding cassette (ABC) transporter family. *Hum Genomics* 2009; **3**: 281-290 [PMID: 19403462 DOI: 10.1186/1479-7364-3-3-281]

31   **Locher KP**. Structure and mechanism of ABC transporters. *Curr Opin Struct Biol* 2004; **14**: 426-431 [PMID: 15313236 DOI: 10.1016/j.sbi.2004.06.005]

32   **Linton KJ**. Structure and function of ABC transporters. *Physiology* (Bethesda) 2007; **22**: 122-130 [PMID: 17420303 DOI: 10.1152/physiol.00046.2006]

33   **Hyde SC**, Emsley P, Hartshorn MJ, Mimmack MM, Gileadi U, Pearce SR, Gallagher MP, Gill DR, Hubbard RE, Higgins CF. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature* 1990; **346**: 362-365 [PMID: 1973824 DOI: 10.1038/346362a0]

34   **Ambudkar SV**, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annu Rev Pharmacol Toxicol* 1999; **39**: 361-398 [PMID: 10331089 DOI: 10.1146/annurev.pharmtox.39.1.361]

35   **Albrecht C**, Viturro E. The ABCA subfamily--gene and protein structures, functions and associated hereditary diseases. *Pflugers Arch* 2007; **453**: 581-589 [PMID: 16586097 DOI: 10.1007/s00424-006-0047-8]

36   **Takahashi K**, Kimura Y, Nagata K, Yamamoto A, Matsuo M, Ueda K. ABC proteins: key molecules for lipid homeostasis. *Med Mol Morphol* 2005; **38**: 2-12 [PMID: 16158173 DOI: 10.1007/s00795-004-0278-8]

37   **Dean M**, Annilo T. Evolution of the ATP-binding cassette (ABC) transporter superfamily in vertebrates. *Annu Rev Genomics Hum Genet* 2005; **6**: 123-142 [PMID: 16124856 DOI: 10.1146/annurev. genom.6.080604.162122]

38   **Esser L**, Zhou F, Pluchino KM, Shiloach J, Ma J, Tang WK, Gutierrez C, Zhang A, Shukla S, Madigan JP, Zhou T, Kwong PD, Ambudkar SV, Gottesman MM, Xia D. Structures of the Multidrug Transporter P-glycoprotein Reveal Asymmetric ATP Binding and the Mechanism of Polyspecificity. *J Biol Chem* 2017; **292**: 446-461 [PMID: 27864369 DOI: 10.1074/jbc.M116.755884]

39   **Callaghan R**. Providing a molecular mechanism for P-glycoprotein; why would I bother? *Biochem Soc Trans* 2015; **43**: 995-1002 [PMID: 26517914 DOI: 10.1042/BST20150131]

40   **Sharom FJ**. The P-glycoprotein multidrug transporter. *Essays Biochem* 2011; **50**: 161-178 [PMID: 21967057 DOI: 10.1042/ bse0500161]

41   **Didziapetris R**, Japertas P, Avdeef A, Petrauskas A. Classification analysis of P-glycoprotein substrate specificity. *J Drug Target* 2003; **11**: 391-406 [PMID: 15203928 DOI: 10.1080/10611860310 001648248]

42   **Ueda K**. ABC proteins protect the human body and maintain optimal health. *Biosci Biotechnol Biochem* 2011; **75**: 401-409 [PMID: 21389634 DOI: 10.1271/bbb.100816]

43   **Soucek P**, Hlavac V, Elsnerova K, Vaclavikova R, Kozevnikovova R, Raus K. Whole exome sequencing analysis of ABCC8 and ABCD2 genes associating with clinical course of breast carcinoma. *Physiol Res* 2015; **64** Suppl 4: S549-S557 [PMID: 26681085]

44   **Cordon-Cardo C**, O'Brien JP, Boccia J, Casals D, Bertino JR, Melamed MR. Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. *J Histochem Cytochem* 1990; **38**: 1277-1287 [PMID: 1974900 DOI: 10.1177/38.9.1974900]

45   **Sarkadi B**, Homolya L, Szakács G, Váradi A. Human multidrug resistance ABCB and ABCG transporters: participation in a chemoimmunity defense system. *Physiol Rev* 2006; **86**: 1179-1236 [PMID: 17015488 DOI: 10.1152/physrev.00037.2005]

46   **Schinkel AH**, Jonker JW. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv Drug Deliv Rev* 2003; **55**: 3-29 [PMID: 12535572 DOI: 10.1016/S0169-409X(02)00169-2]

47   **Kruh GD**, Belinsky MG. The MRP family of drug efflux pumps. *Oncogene* 2003; **22**: 7537-7552 [PMID: 14576857 DOI: 10.1038/ sj.onc.1206953]

48   **Juliano RL**, Ling V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* 1976; **455**: 152-162 [PMID: 990323 DOI: 10.1016/0 005-2736(76)90160-7]

49   **Riordan JR**, Ling V. Purification of P-glycoprotein from plasma membrane vesicles of Chinese hamster ovary cell mutants with reduced colchicine permeability. *J Biol Chem* 1979; **254**: 12701-12705 [PMID: 500733]

50   **Sharom FJ**, Liu R, Qu Q, Romsicki Y. Exploring the structure and function of the P-glycoprotein multidrug transporter using fluorescence spectroscopic tools. *Semin Cell Dev Biol* 2001; **12**: 257-265 [PMID: 11428918 DOI: 10.1006/scdb.2000.0251]

51   **Borgnia MJ**, Eytan GD, Assaraf YG. Competition of hydrophobic peptides, cytotoxic drugs, and chemosensitizers on a common P-glycoprotein pharmacophore as revealed by its ATPase activity. *J Biol Chem* 1996; **271**: 3163-3171 [PMID: 8621716 DOI: 10.1074/ jbc.271.6.3163]

52   **Callaghan R**, Crowley E, Potter S, Kerr ID. P-glycoprotein: so many ways to turn it on. *J Clin Pharmacol* 2008; **48**: 365-378 [PMID: 18156365 DOI: 10.1177/0091270007311568]

53   **Penson RT**, Oliva E, Skates SJ, Glyptis T, Fuller AF Jr, Goodman A, Seiden MV. Expression of multidrug resistance-1 protein inversely correlates with paclitaxel response and survival in ovarian cancer patients: a study in serial samples. *Gynecol Oncol* 2004; **93**: 98-106 [PMID: 15047220 DOI: 10.1016/j.ygyno.2003.11.053]

54   **Han K**, Kahng J, Kim M, Lim J, Kim Y, Cho B, Kim HK, Min WS, Kim CC, Lee KY, Kim BK, Kang CS. Expression of functional

markers in acute nonlymphoblastic leukemia. *Acta Haematol* 2000; **104**: 174-180 [PMID: 11279307 DOI: 10.1159/000046511]

55   **Chung HC**, Rha SY, Kim JH, Roh JK, Min JS, Lee KS, Kim BS, Lee KB. P-glycoprotein: the intermediate end point of drug response to induction chemotherapy in locally advanced breast cancer. *Breast Cancer Res Treat* 1997; **42**: 65-72 [PMID: 9116319 DOI: 10.1023/a:1005739525196]

56   **Teodori E**, Dei S, Martelli C, Scapecchi S, Gualtieri F. The functions and structure of ABC transporters: implications for the design of new inhibitors of Pgp and MRP1 to control multidrug resistance (MDR). *Curr Drug Targets* 2006; **7**: 893-909 [PMID: 16842220 DOI: 10.2174/138945006777709520]

57   **Avendaño C**, Menéndez JC. Inhibitors of multidrug resistance to antitumor agents (MDR). *Curr Med Chem* 2002; **9**: 159-193 [PMID: 11860354 DOI: 10.2174/0929867023371175]

58   **Ueda K**, Cardarelli C, Gottesman MM, Pastan I. Expression of a full-length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc Natl Acad Sci USA* 1987; **84**: 3004-3008 [PMID: 3472246 DOI: 10.1073/ pnas.84.9.3004]

59   **Borst P**, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. *J Natl Cancer Inst* 2000; **92**: 1295-1302 [PMID: 10944550 DOI: 10.1093/ jnci/92.16.1295]

60   **Berger W**, Setinek U, Hollaus P, Zidek T, Steiner E, Elbling L, Cantonati H, Attems J, Gsur A, Micksche M. Multidrug resistance markers P-glycoprotein, multidrug resistance protein 1, and lung resistance protein in non-small cell lung cancer: prognostic implications. *J Cancer Res Clin Oncol* 2005; **131**: 355-363 [PMID: 15856298 DOI: 10.1007/s00432-004-0653-9]

61   **Zurita AJ**, Diestra JE, Condom E, García Del Muro X, Scheffer GL, Scheper RJ, Pérez J, Germà-Lluch JR, Izquierdo MA. Lung resistance-related protein as a predictor of clinical outcome in advanced testicular germ-cell tumours. *Br J Cancer* 2003; **88**: 879-886 [PMID: 12644825 DOI: 10.1038/sj.bjc.6600803]

62   **Kruh GD**, Zeng H, Rea PA, Liu G, Chen ZS, Lee K, Belinsky MG. MRP subfamily transporters and resistance to anticancer agents. *J Bioenerg Biomembr* 2001; **33**: 493-501 [PMID: 11804191 DOI: 10.1023/a:1012827221844]

63   **Schuetz JD**, Connelly MC, Sun D, Paibir SG, Flynn PM, Srinivas RV, Kumar A, Fridland A. MRP4: A previously unidentified factor in resistance to nucleoside-based antiviral drugs. *Nat Med* 1999; **5**: 1048-1051 [PMID: 10470083 DOI: 10.1038/12487]

64   **Reid G**, Wielinga P, Zelcer N, De Haas M, Van Deemter L, Wijnholds J, Balzarini J, Borst P. Characterization of the transport of nucleoside analog drugs by the human multidrug resistance proteins MRP4 and MRP5. *Mol Pharmacol* 2003; **63**: 1094-1103 [PMID: 12695538 DOI: 10.1124/mol.63.5.1094]

65   **Noguchi K**, Katayama K, Mitsuhashi J, Sugimoto Y. Functions of the breast cancer resistance protein (BCRP/ABCG2) in chemotherapy. *Adv Drug Deliv Rev* 2009; **61**: 26-33 [PMID: 19111841 DOI: 10.1016/j.addr.2008.07.003]

66   **Mao Q**, Unadkat JD. Role of the breast cancer resistance protein (ABCG2) in drug transport. *AAPS J* 2005; **7**: E118-E133 [PMID: 16146333 DOI: 10.1208/aapsj070112]

67   **Doyle LA**, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, Ross DD. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci USA* 1998; **95**: 15665-15670 [PMID: 9861027]

68   **Allikmets R**, Schriml LM, Hutchinson A, Romano-Spica V, Dean M. A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. *Cancer Res* 1998; **58**: 5337-5339 [PMID: 9850061]

69   **Scharenberg CW**, Harkey MA, Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood* 2002; **99**: 507-512 [PMID: 11781231 DOI: 10.1182/blood.V99.2.507]

70   **Santamarina-Fojo S**, Remaley AT, Neufeld EB, Brewer HB Jr. Regulation and intracellular trafficking of the ABCA1 transporter.

J Lipid Res 2001; **42**: 1339-1345 [PMID: 11518753]

71   **Oram JF**, Vaughan AM. ABCA1-mediated transport of cellular cholesterol and phospholipids to HDL apolipoproteins. *Curr Opin Lipidol* 2000; **11**: 253-260 [PMID: 10882340]

72   **Ruban EL**, Ferro R, Arifin SA, Falasca M. Lysophosphatidylinositol: a novel link between ABC transporters and G-protein-coupled receptors. *Biochem Soc Trans* 2014; **42**: 1372-1377 [PMID: 25233417 DOI: 10.1042/bst20140151]

73   **Piñeiro R**, Maffucci T, Falasca M. The putative cannabinoid receptor GPR55 defines a novel autocrine loop in cancer cell proliferation. *Oncogene* 2011; **30**: 142-152 [PMID: 20838378 DOI: 10.1038/onc.2010.417]

74   **Mitra P**, Oskeritzian CA, Payne SG, Beaven MA, Milstien S, Spiegel S. Role of ABCC1 in export of sphingosine-1-phosphate from mast cells. *Proc Natl Acad Sci U S A* 2006; **103**: 16394-16399 [PMID: 17050692 DOI: 10.1073/pnas.0603734103]

75   **Fletcher JI**, Haber M, Henderson MJ, Norris MD. ABC transporters in cancer: more than just drug efflux pumps. *Nat Rev Cancer* 2010; **10**: 147-156 [PMID: 20075923 DOI: 10.1038/nrc2789]

76   **König J**, Hartel M, Nies AT, Martignoni ME, Guo J, Büchler MW, Friess H, Keppler D. Expression and localization of human multidrug resistance protein (ABCC) family members in pancreatic carcinoma. *Int J Cancer* 2005; **115**: 359-367 [PMID: 15688370 DOI: 10.1002/ijc.20831]

77   **Scheffer GL**, Kool M, de Haas M, de Vree JM, Pijnenborg AC, Bosman DK, Elferink RP, van der Valk P, Borst P, Scheper RJ. Tissue distribution and induction of human multidrug resistant protein 3. *Lab Invest* 2002; **82**: 193-201 [PMID: 11850532]

78   **Hagmann W**, Jesnowski R, Faissner R, Guo C, Löhr JM. ATP-binding cassette C transporters in human pancreatic carcinoma cell lines. Upregulation in 5-fluorouracil-resistant cells. *Pancreatology* 2009; **9**: 136-144 [PMID: 19077464 DOI: 10.1159/000178884]

79   **Hagmann W**, Faissner R, Schnölzer M, Löhr M, Jesnowski R. Membrane drug transporters and chemoresistance in human pancreatic carcinoma. *Cancers* (Basel) 2010; **3**: 106-125 [PMID: 24212609 DOI: 10.3390/cancers3010106]

80   **Hirohashi T**, Suzuki H, Sugiyama Y. Characterization of the transport properties of cloned rat multidrug resistance-associated protein 3 (MRP3). *J Biol Chem* 1999; **274**: 15181-15185 [PMID: 10329726 DOI: 10.1074/jbc.274.21.15181]

81   **Zeng H**, Liu G, Rea PA, Kruh GD. Transport of amphipathic anions by human multidrug resistance protein 3. *Cancer Res* 2000; **60**: 4779-4784 [PMID: 10987286]

82   **Kool M**, van der Linden M, de Haas M, Scheffer GL, de Vree JM, Smith AJ, Jansen G, Peters GJ, Ponne N, Scheper RJ, Elferink RP, Baas F, Borst P. MRP3, an organic anion transporter able to transport anti-cancer drugs. *Proc Natl Acad Sci USA* 1999; **96**: 6914-6919 [PMID: 10359813 DOI: 10.1073/pnas.96.12.6914]

83   **Hagmann W**, Jesnowski R, Löhr JM. Interdependence of gemcitabine treatment, transporter expression, and resistance in human pancreatic carcinoma cells. *Neoplasia* 2010; **12**: 740-747 [PMID: 20824050 DOI: 10.1593/neo.10576]

84   **Nambaru PK**, Hübner T, Köck K, Mews S, Grube M, Payen L, Guitton J, Sendler M, Jedlitschky G, Rimmbach C, Rosskopf D, Kowalczyk DW, Kroemer HK, Weiss FU, Mayerle J, Lerch MM, Ritter CA. Drug efflux transporter multidrug resistance-associated protein 5 affects sensitivity of pancreatic cancer cell lines to the nucleoside anticancer drug 5-fluorouracil. *Drug Metab Dispos* 2011; **39**: 132-139 [PMID: 20930123 DOI: 10.1124/dmd.110.033613]

85   **Falasca M**, Kim M, Casari I. Pancreatic cancer: Current research and future directions. *Biochim Biophys Acta* 2016; **1865**: 123-132 [PMID: 26794394 DOI: 10.1016/j.bbcan.2016.01.001]

86   **Hanahan D**, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]

87   **Sousa CM**, Kimmelman AC. The complex landscape of pancreatic cancer metabolism. *Carcinogenesis* 2014; **35**: 1441-1450 [PMID: 24743516 DOI: 10.1093/carcin/bgu097]

88   **Ying H**, Kimmelman AC, Lyssiotis CA, Hua S, Chu GC, Fletcher-Sananikone E, Locasale JW, Son J, Zhang H, Coloff JL, Yan H, Wang W, Chen S, Viale A, Zheng H, Paik JH, Lim C, Guimaraes AR, Martin ES, Chang J, Hezel AF, Perry SR, Hu J, Gan B, Xiao Y, Asara JM, Weissleder R, Wang YA, Chin L, Cantley LC, DePinho RA. Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. *Cell* 2012; **149**: 656-670 [PMID: 22541435 DOI: 10.1016/j.cell.2012.01.058]

89   **Son J**, Lyssiotis CA, Ying H, Wang X, Hua S, Ligorio M, Perera RM, Ferrone CR, Mullarky E, Shyh-Chang N, Kang Y, Fleming JB, Bardeesy N, Asara JM, Haigis MC, DePinho RA, Cantley LC, Kimmelman AC. Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. *Nature* 2013; **496**: 101-105 [PMID: 23535601 DOI: 10.1038/nature12040]

90   **Swierczynski J**, Hebanowska A, Sledzinski T. Role of abnormal lipid metabolism in development, progression, diagnosis and therapy of pancreatic cancer. *World J Gastroenterol* 2014; **20**: 2279-2303 [PMID: 24605027 DOI: 10.3748/wjg.v20.i9.2279]

91   **Kamphorst JJ**, Cross JR, Fan J, de Stanchina E, Mathew R, White EP, Thompson CB, Rabinowitz JD. Hypoxic and Ras-transformed cells support growth by scavenging unsaturated fatty acids from lysophospholipids. *Proc Natl Acad Sci USA* 2013; **110**: 8882-8887 [PMID: 23671091 DOI: 10.1073/pnas.1307237110]

92   **Rysman E**, Brusselmans K, Scheys K, Timmermans L, Derua R, Munck S, Van Veldhoven PP, Waltregny D, Daniëls VW, Machiels J, Vanderhoydonc F, Smans K, Waelkens E, Verhoeven G, Swinnen JV. De novo lipogenesis protects cancer cells from free radicals and chemotherapeutics by promoting membrane lipid saturation. *Cancer Res* 2010; **70**: 8117-8126 [PMID: 20876798 DOI: 10.1158/0008-5472.can-09-3871]

93   **Baenke F**, Peck B, Miess H, Schulze A. Hooked on fat: the role of lipid synthesis in cancer metabolism and tumour development. *Dis Model Mech* 2013; **6**: 1353-1363 [PMID: 24203995 DOI: 10.1242/dmm.011338]

94   **Guillaumond F**, Bidaut G, Ouaissi M, Servais S, Gouirand V, Olivares O, Lac S, Borge L, Roques J, Gayet O, Pinault M, Guimaraes C, Nigri J, Loncle C, Lavaut MN, Garcia S, Tailleux A, Staels B, Calvo E, Tomasini R, Iovanna JL, Vasseur S. Cholesterol uptake disruption, in association with chemotherapy, is a promising combined metabolic therapy for pancreatic adenocarcinoma. *Proc Natl Acad Sci USA* 2015; **112**: 2473-2478 [PMID: 25675507 DOI: 10.1073/pnas.1421601112]

95   **Mohelnikova-Duchonova B**, Brynychova V, Oliverius M, Honsova E, Kala Z, Muckova K, Soucek P. Differences in transcript levels of ABC transporters between pancreatic adenocarcinoma and nonneoplastic tissues. *Pancreas* 2013; **42**: 707-716 [PMID: 23462326 DOI: 10.1097/MPA.0b013e318279b861]

96   **Mantovani A**, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* 2008; **454**: 436-444 [PMID: 18650914 DOI: 10.1038/nature07205]

97   **Tessner TG**, Muhale F, Riehl TE, Anant S, Stenson WF. Prostaglandin E2 reduces radiation-induced epithelial apoptosis through a mechanism involving AKT activation and bax translocation. *J Clin Invest* 2004; **114**: 1676-1685 [PMID: 15578100 DOI: 10.1172/jci22218]

98   **Reid G**, Wielinga P, Zelcer N, van der Heijden I, Kuil A, de Haas M, Wijnholds J, Borst P. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. *Proc Natl Acad Sci USA* 2003; **100**: 9244-9249 [PMID: 12835412 DOI: 10.1073/pnas.1033060100]

99   **Knab LM**, Grippo PJ, Bentrem DJ. Involvement of eicosanoids in the pathogenesis of pancreatic cancer: the roles of cyclooxygenase-2 and 5-lipoxygenase. *World J Gastroenterol* 2014; **20**: 10729-10739 [PMID: 25152576 DOI: 10.3748/wjg.v20.i31.10729]

100   **Hussain SP**, Hofseth LJ, Harris CC. Radical causes of cancer. *Nat Rev Cancer* 2003; **3**: 276-285 [PMID: 12671666 DOI: 10.1038/nrc1046]

101   **Meister A**. Selective modification of glutathione metabolism. *Science* 1983; **220**: 472-477 [PMID: 6836290 DOI: 10.1126/

102   **Liesa M**, Qiu W, Shirihai OS. Mitochondrial ABC transporters function: the role of ABCB10 (ABC-me) as a novel player in cellular handling of reactive oxygen species. *Biochim Biophys Acta* 2012; **1823**: 1945-1957 [PMID: 22884976 DOI: 10.1016/j.bbamcr.2012.07.013]

103   **Demicco EG**, Kavanagh KT, Romieu-Mourez R, Wang X, Shin SR, Landesman-Bollag E, Seldin DC, Sonenshein GE. RelB/p52 NF-kappaB complexes rescue an early delay in mammary gland development in transgenic mice with targeted superrepressor IkappaB-alpha expression and promote carcinogenesis of the mammary gland. *Mol Cell Biol* 2005; **25**: 10136-10147 [PMID: 16260626 DOI: 10.1128/mcb.25.22.10136-10147.2005]

104   **Wang X**, Campos CR, Peart JC, Smith LK, Boni JL, Cannon RE, Miller DS. Nrf2 upregulates ATP binding cassette transporter expression and activity at the blood-brain and blood-spinal cord barriers. *J Neurosci* 2014; **34**: 8585-8593 [PMID: 24948812 DOI: 10.1523/jneurosci.2935-13.2014]

105   **Cabello CM**, Bair WB 3rd, Wondrak GT. Experimental therapeutics: targeting the redox Achilles heel of cancer. *Curr Opin Investig Drugs* 2007; **8**: 1022-1037 [PMID: 18058573]

106   **Olive KP**, Jacobetz MA, Davidson CJ, Gopinathan A, McIntyre D, Honess D, Madhu B, Goldgraben MA, Caldwell ME, Allard D, Frese KK, Denicola G, Feig C, Combs C, Winter SP, Ireland-Zecchini H, Reichelt S, Howat WJ, Chang A, Dhara M, Wang L, Rückert F, Grützmann R, Pilarsky C, Izeradjene K, Hingorani SR, Huang P, Davies SE, Plunkett W, Egorin M, Hruban RH, Whitebread N, McGovern K, Adams J, Iacobuzio-Donahue C, Griffiths J, Tuveson DA. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science* 2009; **324**: 1457-1461 [PMID: 19460966 DOI: 10.1126/science.1171362]

107   Olive K. Clinical Trial: IPI-926-03 for metastatic pancreatic ductal adenocarcinoma patients who have not been treated with other chemotherapy. Available from: https://www.olivelab.org/ipi-926-03.html. Accessed on: 17.04.2018

108   **Hingorani SR**, Harris WP, Beck JT, Berdov BA, Wagner SA, Pshevlotsky EM, Tjulandin SA, Gladkov OA, Holcombe RF, Korn R, Raghunand N, Dychter S, Jiang P, Shepard HM, Devoe CE. Phase Ib Study of PEGylated Recombinant Human Hyaluronidase and Gemcitabine in Patients with Advanced Pancreatic Cancer. *Clin Cancer Res* 2016; **22**: 2848-2854 [PMID: 26813359 DOI: 10.1158/1078-0432.ccr-15-2010]

109   **Moreau A**, Le Vee M, Jouan E, Parmentier Y, Fardel O. Drug transporter expression in human macrophages. *Fundam Clin Pharmacol* 2011; **25**: 743-752 [PMID: 21210849 DOI: 10.1111/j.1472-8206.2010.00913.x]

110   **Vander Borght S**, Komuta M, Libbrecht L, Katoonizadeh A, Aerts R, Dymarkowski S, Verslype C, Nevens F, Roskams T. Expression of multidrug resistance-associated protein 1 in hepatocellular carcinoma is associated with a more aggressive tumour phenotype and may reflect a progenitor cell origin. *Liver Int* 2008; **28**: 1370-1380 [PMID: 19055643 DOI: 10.1111/j.1478-3231.2008.01889.x]

111   **Weinstein RS**, Jakate SM, Dominguez JM, Lebovitz MD, Koukoulis GK, Kuszak JR, Klusens LF, Grogan TM, Saclarides TJ, Roninson IB. Relationship of the expression of the multidrug resistance gene product (P-glycoprotein) in human colon carcinoma to local tumor aggressiveness and lymph node metastasis. *Cancer Res* 1991; **51**: 2720-2726 [PMID: 1673639]

112   **Filipits M**, Suchomel RW, Dekan G, Haider K, Valdimarsson G, Depisch D, Pirker R. MRP and MDR1 gene expression in primary breast carcinomas. *Clin Cancer Res* 1996; **2**: 1231-1237 [PMID: 9816292]

113   **Lee CJ**, Li C, Simeone DM. Human pancreatic cancer stem cells: implications for how we treat pancreatic cancer. *Transl Oncol* 2008; **1**: 14-18 [PMID: 18607507 DOI: 10.1593/tlo.08013]

114   **Li C**, Lee CJ, Simeone DM. Identification of human pancreatic cancer stem cells. *Methods Mol Biol* 2009; **568**: 161-173 [PMID: 19582426 DOI: 10.1007/978-1-59745-280-9_10]

115   **Dean M**, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nat Rev Cancer* 2005; **5**: 275-284 [PMID: 15803154 DOI: 10.1038/nrc1590]

116   **Barbet R**, Peiffer I, Hutchins JR, Hatzfeld A, Garrido E, Hatzfeld JA. Expression of the 49 human ATP binding cassette (ABC) genes in pluripotent embryonic stem cells and in early- and late-stage multipotent mesenchymal stem cells: possible role of ABC plasma membrane transporters in maintaining human stem cell pluripotency. *Cell Cycle* 2012; **11**: 1611-1620 [PMID: 22456339 DOI: 10.4161/cc.20023]

117   The Human Protein Atlas. Available from: https://www.proteinatlas.org/. Accessed on 18.04.2018

118   **Dvorak P**, Pesta M, Soucek P. ABC gene expression profiles have clinical importance and possibly form a new hallmark of cancer. *Tumour Biol* 2017; **39**: 1010428317699800 [PMID: 28468577 DOI: 10.1177/1010428317699800]

119   **Dvorak P**, Hlavac V, Mohelnikova-Duchonova B, Liska V, Pesta M, Soucek P. Downregulation of ABC Transporters in Non-neoplastic Tissues Confers Better Prognosis for Pancreatic and Colorectal Cancer Patients. *J Cancer* 2017; **8**: 1959-1971 [PMID: 28819395 DOI: 10.7150/jca.19364]

120   **Kathawala RJ**, Gupta P, Ashby CR Jr, Chen ZS. The modulation of ABC transporter-mediated multidrug resistance in cancer: a review of the past decade. *Drug Resist Updat* 2015; **18**: 1-17 [PMID: 25554624 DOI: 10.1016/j.drup.2014.11.002]

121   **Falasca M**, Linton KJ. Investigational ABC transporter inhibitors. *Expert Opin Investig Drugs* 2012; **21**: 657-666 [PMID: 22493979 DOI: 10.1517/13543784.2012.679339]

122   **Tsuruo T**, Iida H, Tsukagoshi S, Sakurai Y. Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res* 1981; **41**: 1967-1972 [PMID: 7214365]

123   **Kelly RJ**, Draper D, Chen CC, Robey RW, Figg WD, Piekarz RL, Chen X, Gardner ER, Balis FM, Venkatesan AM, Steinberg SM, Fojo T, Bates SE. A pharmacodynamic study of docetaxel in combination with the P-glycoprotein antagonist tariquidar (XR9576) in patients with lung, ovarian, and cervical cancer. *Clin Cancer Res* 2011; **17**: 569-580 [PMID: 21081657 DOI: 10.1158/1078-0432.ccr-10-1725]

124   **Pennock GD**, Dalton WS, Roeske WR, Appleton CP, Mosley K, Plezia P, Miller TP, Salmon SE. Systemic toxic effects associated with high-dose verapamil infusion and chemotherapy administration. *J Natl Cancer Inst* 1991; **83**: 105-110 [PMID: 1988684 DOI: 10.1093/jnci/83.2.105]

125   **Yu M**, Ocana A, Tannock IF. Reversal of ATP-binding cassette drug transporter activity to modulate chemoresistance: why has it failed to provide clinical benefit? *Cancer Metastasis Rev* 2013; **32**: 211-227 [PMID: 23093326 DOI: 10.1007/s10555-012-9402-8]

126   **Tidefelt U**, Liliemark J, Gruber A, Liliemark E, Sundman-Engberg B, Juliusson G, Stenke L, Elmhorn-Rosenborg A, Möllgård L, Lehman S, Xu D, Covelli A, Gustavsson B, Paul C. P-Glycoprotein inhibitor valspodar (PSC 833) increases the intracellular concentrations of daunorubicin in vivo in patients with P-glycoprotein-positive acute myeloid leukemia. *J Clin Oncol* 2000; **18**: 1837-1844 [PMID: 10784624 DOI: 10.1200/jco.2000.18.9.1837]

127   **Lhommé C**, Joly F, Walker JL, Lissoni AA, Nicoletto MO, Manikhas GM, Baekelandt MM, Gordon AN, Fracasso PM, Mietlowski WL, Jones GJ, Dugan MH. Phase III study of valspodar (PSC 833) combined with paclitaxel and carboplatin compared with paclitaxel and carboplatin alone in patients with stage IV or suboptimally debulked stage III epithelial ovarian cancer or primary peritoneal cancer. *J Clin Oncol* 2008; **26**: 2674-2682 [PMID: 18509179 DOI: 10.1200/jco.2007.14.9807]

128   **Greenberg PL**, Lee SJ, Advani R, Tallman MS, Sikic BI, Letendre L, Dugan K, Lum B, Chin DL, Dewald G, Paietta E, Bennett JM, Rowe JM. Mitoxantrone, etoposide, and cytarabine with or without valspodar in patients with relapsed or refractory acute myeloid leukemia and high-risk myelodysplastic syndrome: a phase III trial (E2995). *J Clin Oncol* 2004; **22**: 1078-1086 [PMID: 15020609

DOI: 10.1200/jco.2004.07.048]

129 **Minderman H**, O'Loughlin KL, Pendyala L, Baer MR. VX-710 (biricodar) increases drug retention and enhances chemosensitivity in resistant cells overexpressing P-glycoprotein, multidrug resistance protein, and breast cancer resistance protein. *Clin Cancer Res* 2004; **10**: 1826-1834 [PMID: 15014037 DOI: 10.1158/1078-0432.CCR-0914-3]

130 **Gottesman MM**, Ludwig J, Xia D, Szakács G. Defeating drug resistance in cancer. *Discov Med* 2006; **6**: 18-23 [PMID: 17234123]

131 **Tang R**, Faussat AM, Perrot JY, Marjanovic Z, Cohen S, Storme T, Morjani H, Legrand O, Marie JP. Zosuquidar restores drug sensitivity in P-glycoprotein expressing acute myeloid leukemia (AML). *BMC Cancer* 2008; **8**: 51 [PMID: 18271955 DOI: 10.1186/1471-2407-8-51]

132 **Kemper EM**, Cleypool C, Boogerd W, Beijnen JH, van Tellingen O. The influence of the P-glycoprotein inhibitor zosuquidar trihydrochloride (LY335979) on the brain penetration of paclitaxel in mice. *Cancer Chemother Pharmacol* 2004; **53**: 173-178 [PMID: 14605863 DOI: 10.1007/s00280-003-0720-y]

133 **Dörner B**, Kuntner C, Bankstahl JP, Bankstahl M, Stanek J, Wanek T, Stundner G, Mairinger S, Löscher W, Müller M, Langer O, Erker T. Synthesis and small-animal positron emission tomography evaluation of [11C]-elacridar as a radiotracer to assess the distribution of P-glycoprotein at the blood-brain barrier. *J Med Chem* 2009; **52**: 6073-6082 [PMID: 19711894 DOI: 10.1021/jm900940f]

134 **Bates SE**, Shieh CY, Mickley LA, Dichek HL, Gazdar A, Loriaux DL, Fojo AT. Mitotane enhances cytotoxicity of chemotherapy in cell lines expressing a multidrug resistance gene (mdr-1/P-glycoprotein) which is also expressed by adrenocortical carcinomas. *J Clin Endocrinol Metab* 1991; **73**: 18-29 [PMID: 1675220 DOI: 10.1210/jcem-73-1-18]

135 **Consoli U**, Priebe W, Ling YH, Mahadevia R, Griffin M, Zhao S, Perez-Soler R, Andreeff M. The novel anthracycline annamycin is not affected by P-glycoprotein-related multidrug resistance: comparison with idarubicin and doxorubicin in HL-60 leukemia cell lines. *Blood* 1996; **88**: 633-644 [PMID: 8695811]

136 **Fox E**, Bates SE. Tariquidar (XR9576): a P-glycoprotein drug efflux pump inhibitor. *Expert Rev Anticancer Ther* 2007; **7**: 447-459 [PMID: 17428165 DOI: 10.1586/14737140.7.4.447]

137 **Patel J**, Mitra AK. Strategies to overcome simultaneous P-glycoprotein mediated efflux and CYP3A4 mediated metabolism of drugs. *Pharmacogenomics* 2001; **2**: 401-415 [PMID: 11722289 DOI: 10.1517/14622416.2.4.401]

138 **Fromm MF**. Importance of P-glycoprotein at blood-tissue barriers. *Trends Pharmacol Sci* 2004; **25**: 423-429 [PMID: 15276711 DOI: 10.1016/j.tips.2004.06.002]

139 **van de Ven R**, Scheffer GL, Scheper RJ, de Gruijl TD. The ABC of dendritic cell development and function. *Trends Immunol* 2009; **30**: 421-429 [PMID: 19699682 DOI: 10.1016/j.it.2009.06.004]

140 **Gekeler V**, Ise W, Sanders KH, Ulrich WR, Beck J. The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. *Biochem Biophys Res Commun* 1995; **208**: 345-352 [PMID: 7887949 DOI: 10.1006/bbrc.1995.1344]

141 **Kim HS**, Min YD, Choi CH. Double-edged sword of chemosensitizer: increase of multidrug resistance protein (MRP) in leukemic cells by an MRP inhibitor probenecid. *Biochem Biophys Res Commun* 2001; **283**: 64-71 [PMID: 11322768 DOI: 10.1006/bbrc.2001.4746]

142 **O'Connor R**, O'Leary M, Ballot J, Collins CD, Kinsella P, Mager DE, Arnold RD, O'Driscoll L, Larkin A, Kennedy S, Fennelly D, Clynes M, Crown J. A phase I clinical and pharmacokinetic study of the multi-drug resistance protein-1 (MRP-1) inhibitor sulindac, in combination with epirubicin in patients with advanced cancer. *Cancer Chemother Pharmacol* 2007; **59**: 79-87 [PMID: 16642371 DOI: 10.1007/s00280-006-0240-7]

143 **Rabindran SK**, Ross DD, Doyle LA, Yang W, Greenberger LM. Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. *Cancer Res* 2000; **60**: 47-50 [PMID: 10646850]

144 **Tiwari AK**, Sodani K, Dai CL, Ashby CR Jr, Chen ZS. Revisiting the ABCs of multidrug resistance in cancer chemotherapy. *Curr Pharm Biotechnol* 2011; **12**: 570-594 [PMID: 21118094 DOI: 10.2174/138920111795164048]

145 **Zhang YK**, Wang YJ, Gupta P, Chen ZS. Multidrug Resistance Proteins (MRPs) and Cancer Therapy. *AAPS J* 2015; **17**: 802-812 [PMID: 25840885 DOI: 10.1208/s12248-015-9757-1]

146 **Zhou SF**, Wang LL, Di YM, Xue CC, Duan W, Li CG, Li Y. Substrates and inhibitors of human multidrug resistance associated proteins and the implications in drug development. *Curr Med Chem* 2008; **15**: 1981-2039 [PMID: 18691054 DOI: 10.2174/092986708785132870]

147 **Widmer N**, Rumpold H, Untergasser G, Fayet A, Buclin T, Decosterd LA. Resistance reversal by RNAi silencing of MDR1 in CML cells associated with increase in imatinib intracellular levels. *Leukemia* 2007; **21**: 1561-1562; author reply 1562-1564 [PMID: 17429432 DOI: 10.1038/sj.leu.2404671]

148 **Mechetner EB**, Roninson IB. Efficient inhibition of P-glycoprotein-mediated multidrug resistance with a monoclonal antibody. *Proc Natl Acad Sci USA* 1992; **89**: 5824-5828 [PMID: 1352877 DOI: 10.1073/pnas.89.13.5824]

149 **Fisher M**, Abramov M, Van Aerschot A, Xu D, Juliano RL, Herdewijn P. Inhibition of MDR1 expression with altritol-modified siRNAs. *Nucleic Acids Res* 2007; **35**: 1064-1074 [PMID: 17264131 DOI: 10.1093/nar/gkl1126]

150 **Wu H**, Hait WN, Yang JM. Small interfering RNA-induced suppression of MDR1 (P-glycoprotein) restores sensitivity to multidrug-resistant cancer cells. *Cancer Res* 2003; **63**: 1515-1519 [PMID: 12670898]

151 **Wang Z**, Li Y, Ahmad A, Azmi AS, Kong D, Banerjee S, Sarkar FH. Targeting miRNAs involved in cancer stem cell and EMT regulation: An emerging concept in overcoming drug resistance. *Drug Resist Updat* 2010; **13**: 109-118 [PMID: 20692200 DOI: 10.1016/j.drup.2010.07.001]

152 **Ma J**, Dong C, Ji C. MicroRNA and drug resistance. *Cancer Gene Ther* 2010; **17**: 523-531 [PMID: 20467450 DOI: 10.1038/cgt.2010.18]

153 **Giovannetti E**, Funel N, Peters GJ, Del Chiaro M, Erozenci LA, Vasile E, Leon LG, Pollina LE, Groen A, Falcone A, Danesi R, Campani D, Verheul HM, Boggi U. MicroRNA-21 in pancreatic cancer: correlation with clinical outcome and pharmacologic aspects underlying its role in the modulation of gemcitabine activity. *Cancer Res* 2010; **70**: 4528-4538 [PMID: 20460539 DOI: 10.1158/0008-5472.can-09-4467]

154 **Zhu H**, Wu H, Liu X, Evans BR, Medina DJ, Liu CG, Yang JM. Role of MicroRNA miR-27a and miR-451 in the regulation of MDR1/P-glycoprotein expression in human cancer cells. *Biochem Pharmacol* 2008; **76**: 582-588 [PMID: 18619946 DOI: 10.1016/j.bcp.2008.06.007]

155 **Wang F**, Xue X, Wei J, An Y, Yao J, Cai H, Wu J, Dai C, Qian Z, Xu Z, Miao Y. hsa-miR-520h downregulates ABCG2 in pancreatic cancer cells to inhibit migration, invasion, and side populations. *Br J Cancer* 2010; **103**: 567-574 [PMID: 20628378 DOI: 10.1038/sj.bjc.6605724]

156 **Hwang JH**, Voortman J, Giovannetti E, Steinberg SM, Leon LG, Kim YT, Funel N, Park JK, Kim MA, Kang GH, Kim SW, Del Chiaro M, Peters GJ, Giaccone G. Identification of microRNA-21 as a biomarker for chemoresistance and clinical outcome following adjuvant therapy in resectable pancreatic cancer. *PLoS One* 2010; **5**: e10630 [PMID: 20498843 DOI: 10.1371/journal.pone.0010630]

157 **Ozvegy-Laczka C**, Cserepes J, Elkind NB, Sarkadi B. Tyrosine kinase inhibitor resistance in cancer: role of ABC multidrug transporters. *Drug Resist Updat* 2005; **8**: 15-26 [PMID: 15939339 DOI: 10.1016/j.drup.2005.02.002]

158 **Brendel C**, Scharenberg C, Dohse M, Robey RW, Bates SE, Shukla S, Ambudkar SV, Wang Y, Wennemuth G, Burchert A, Boudriot U, Neubauer A. Imatinib mesylate and nilotinib (AMN107) exhibit high-affinity interaction with ABCG2 on primitive hematopoietic

stem cells. *Leukemia* 2007; **21**: 1267-1275 [PMID: 17519960 DOI: 10.1038/sj.leu.2404638]

159 **Dai CL**, Liang YJ, Wang YS, Tiwari AK, Yan YY, Wang F, Chen ZS, Tong XZ, Fu LW. Sensitization of ABCG2-overexpressing cells to conventional chemotherapeutic agent by sunitinib was associated with inhibiting the function of ABCG2. *Cancer Lett* 2009; **279**: 74-83 [PMID: 19232821 DOI: 10.1016/j.canlet.2009.01.027]

160 **Dai CL**, Tiwari AK, Wu CP, Su XD, Wang SR, Liu DG, Ashby CR Jr, Huang Y, Robey RW, Liang YJ, Chen LM, Shi CJ, Ambudkar SV, Chen ZS, Fu LW. Lapatinib (Tykerb, GW572016) reverses multidrug resistance in cancer cells by inhibiting the activity of ATP-binding cassette subfamily B member 1 and G member 2. *Cancer Res* 2008; **68**: 7905-7914 [PMID: 18829547 DOI: 10.1158/0008-5472.can-08-0499]

161 **Kuang YH**, Shen T, Chen X, Sodani K, Hopper-Borge E, Tiwari AK, Lee JW, Fu LW, Chen ZS. Lapatinib and erlotinib are potent reversal agents for MRP7 (ABCC10)-mediated multidrug resistance. *Biochem Pharmacol* 2010; **79**: 154-161 [PMID: 19720054 DOI: 10.1016/j.bcp.2009.08.021]

162 **Livney YD**, Assaraf YG. Rationally designed nanovehicles to overcome cancer chemoresistance. *Adv Drug Deliv Rev* 2013; **65**: 1716-1730 [PMID: 23954781 DOI: 10.1016/j.addr.2013.08.006]

163 **Sun T**, Zhang YS, Pang B, Hyun DC, Yang M, Xia Y. Engineered nanoparticles for drug delivery in cancer therapy. *Angew Chem Int Ed Engl* 2014; **53**: 12320-12364 [PMID: 25294565 DOI: 10.1002/anie.201403036]

164 **Xue X**, Liang XJ. Overcoming drug efflux-based multidrug resistance in cancer with nanotechnology. *Chin J Cancer* 2012; **31**: 100-109 [PMID: 22237039 DOI: 10.5732/cjc.011.10326]

165 **Kool M**, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJ, Juijn JA, Baas F, Borst P. Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Res* 1997; **57**: 3537-3547 [PMID: 9270026]

166 **Kaye SB**. Reversal of drug resistance in ovarian cancer: where do we go from here? *J Clin Oncol* 2008; **26**: 2616-2618 [PMID: 18509172 DOI: 10.1200/jco.2008.16.2123]

167 **Relling MV**. Are the major effects of P-glycoprotein modulators due to altered pharmacokinetics of anticancer drugs? *Ther Drug Monit* 1996; **18**: 350-356 [PMID: 8857549]

168 **Ozols RF**, Cunnion RE, Klecker RW Jr, Hamilton TC, Ostchega Y, Parrillo JE, Young RC. Verapamil and adriamycin in the treatment of drug-resistant ovarian cancer patients. *J Clin Oncol* 1987; **5**: 641-647 [PMID: 3559654 DOI: 10.1200/jco.1987.5.4.641]

169 **Park S**, Shimizu C, Shimoyama T, Takeda M, Ando M, Kohno T, Katsumata N, Kang YK, Nishio K, Fujiwara Y. Gene expression profiling of ATP-binding cassette (ABC) transporters as a predictor of the pathologic response to neoadjuvant chemotherapy in breast cancer patients. *Breast Cancer Res Treat* 2006; **99**: 9-17 [PMID: 16752223 DOI: 10.1007/s10549-006-9175-2]

170 **Marzac C**, Garrido E, Tang R, Fava F, Hirsch P, De Benedictis C, Corre E, Lapusan S, Lallemand JY, Marie JP, Jacquet E, Legrand O. ATP Binding Cassette transporters associated with chemoresistance: transcriptional profiling in extreme cohorts and their prognostic impact in a cohort of 281 acute myeloid leukemia patients. *Haematologica* 2011; **96**: 1293-1301 [PMID: 21606172 DOI: 10.3324/haematol.2010.031823]

171 **Chen Z**, Shi T, Zhang L, Zhu P, Deng M, Huang C, Hu T, Jiang L, Li J. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family in multidrug resistance: A review of the past decade. *Cancer Lett* 2016; **370**: 153-164 [PMID: 26499806 DOI: 10.1016/j.canlet.2015.10.010]

172 **Kim RB**. Drugs as P-glycoprotein substrates, inhibitors, and inducers. *Drug Metab Rev* 2002; **34**: 47-54 [PMID: 11996011 DOI: 10.1081/dmr-120001389]

173 **Slot AJ**, Molinski SV, Cole SP. Mammalian multidrug-resistance proteins (MRPs). *Essays Biochem* 2011; **50**: 179-207 [PMID: 21967058 DOI: 10.1042/bse0500179]

MINIREVIEWS

Rethinking *de novo* immune hepatitis, an old concept for liver allograft rejection: Relevance of glutathione S-transferase T1 mismatch

Isabel Aguilera, Elena Aguado-Dominguez, Jose Manuel Sousa, Antonio Nuñez-Roldan

Isabel Aguilera, Elena Aguado-Dominguez, Antonio Nuñez-Roldan, Department of Immunology, Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocío, Sevilla 41013, Spain

Jose Manuel Sousa, Digestive and Liver Diseases Service, Hospital Universitario Virgen del Rocío, Sevilla 41013, Spain

ORCID number: Isabel Aguilera (0000-0003-2165-4954); Elena Aguado-Dominguez (0000-0003-1519-6002); Jose Manuel Sousa (0000-0002-8158-273X); Antonio Nuñez-Roldan (0000-0001-6479-2586).

Correspondence to: Isabel Aguilera, PhD, Research Fellow, Senior Scientist, Department of Immunology, Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocío, Avda Manuel Siurot s/n, Sevilla 41013, Spain. iaguilera-ibis@us.es
Telephone: +34-955923120

Abstract

Antibody-mediated rejection (AMR) in liver transplantation has long been underestimated. The concept of the liver as an organ susceptible to AMR has emerged in recent years, not only in the context of the major histocompatibility complex with the presence of HLA donor-specific antibodies, but also with antigens regarded as "minor", whose role in AMR has been demonstrated. Among them, antibodies against glutathione S-transferase T1 have been found in 100% of patients with *de novo* autoimmune hepatitis (dnAIH) when studied. In its latest update, the Banff Working Group for liver allograft pathology proposed replacing the term dnAIH with plasma cell (PC)-rich rejection. Antibodies to glutathione S-transferase T1 (GSTT1) in null recipients of GSTT1 positive donors have been included as a contributory but nonessential feature of the diagnosis of PC-rich rejection. Also in this update, non-organ-specific anti-nuclear or smooth muscle autoantibodies are no longer included as diagnostic criteria. Although initially found in a proportion of patients with PC-rich rejection, the presence of autoantibodies is misleading since they are not disease-specific and appear in many different contexts as bystanders. The cellular types and proportions of the inflammatory infiltrates in diagnostic biopsies have been studied in detail very recently. PC-rich rejection

biopsies present a characteristic cellular profile with a predominance of T lymphocytes and a high proportion of PCs, close to 30%, of which 16.48% are IgG4^{+}. New data on the relevance of GSTT1-specific T lymphocytes to PC-rich rejection will be discussed in this review.

Key words: Glutathione S-transferase T1 mismatch; Liver allograft rejection; Plasma cell-rich rejection; *De novo* autoimmune hepatitis; Donor-specific antibodies; NewCAST; Cell quantification; IgG4^{+} plasma cell; T lymphocytes

Core tip: The purpose of this review is to update the reader with recent knowledge about a disease of the liver allograft, whose definition has evolved from "*de novo* autoimmune hepatitis" to "plasma cell-rich rejection". During the last 20 years, several groups have contributed new data that has prompted the liver transplant community to reconsider several aspects of the disease. It is not the intention of this review to go over details of the histological features or the role of autoantibodies in this disease, which have been well described in other reviews. Instead, more recent aspects, such as the composition of infiltrates in biopsies and T cell involvement will be discussed.

INTRODUCTION

Antibody-mediated rejection (AMR) in liver transplantation is becoming increasingly relevant after being considered an immune privileged organ for many years. Indeed, a good HLA match between donor and recipient - very important in other settings such as kidney transplants - was never considered as essential in liver donations. A few years ago, a number of publications describing a pathogenic role for HLA donor-specific antibodies (DSA) came out indicating that the liver was prone to experience AMR like any other organ[1]. Earlier, in 1998, a new liver transplant-associated disease termed *de novo* autoimmune hepatitis (dnAIH) was described[2] and many groups reported cases of patients with similar characteristics but with different prevalence[3-25]. The diagnostic criteria are well described, particularly with regard to histological features that are essential for differential diagnosis in adult[26-32] and pediatric[9,33] cases. To complete the characterization of this special type of immune response, now generally accepted as rejection

but with disconcerting similarities with autoimmunity[34], there are a few aspects that still need to be investigated.

For example, one important issue that has yet to be addressed is the role of allelic disparity of glutathione S-transferase T1 (GSTT1) or other minor histocompatibility antigen mismatches in the development of dnAIH in pediatric liver transplant. There are very few studies about the long-term consequences of dnAIH in the liver allograft of children. Ekong *et al*[14] reported their observations from a retrospective multi-center study that included 29 children from 5 centers. The authors showed that half of the patients did not experience rejection prior to diagnosis and the response to steroid therapy was good in general but not in all the cases. Interestingly, 38% of the children had abnormal liver enzymes over 2-fold the upper limit of normal, especially gamma-glutamyltransferase (GGT) at the time of last follow-up, indicating bile duct injury. This result contradicts one of the main arguments against considering dnAIH a type of rejection, namely the absence of bile duct involvement.

Well-established immunological criteria for diagnosing AMR in kidney transplantation include detection of complement component 4d (C4d) deposits in peritubular capillaries concomitantly with antidonor serology[35]. Presently, C4d deposition in portal capillaries is accepted as a distinctive feature of dnAIH/PC hepatitis[36,37] although it is not currently considered to be a diagnostic criterion.

IgG4 has been traditionally considered a benign antibody although this concept has changed due to the growing number of IgG4-related diseases described in the literature during the last few years. International experts in the field held a symposium in Boston in 2012 and generated consensus guidelines for the diagnosis of IgG4-related diseases[38]. Since an important presence of IgG4^{+} plasma cells (PCs) has been detected in subgroups of patients with dnAIH/PC-rich rejection, this aspect will be discussed later in this review.

TERMINOLOGY CONTROVERSY

When pathologists first identified the transplant-associated pathology dnAIH, the histological features described were very similar to those found in autoimmune hepatitis[2]. Following this first study in 1998[2], dnAIH became the term of choice[6-8,11,12,17,22,25,39,40]. However, from the beginning, clinicians found it difficult to admit progression to autoimmunity in a patient's graft without a previous history of autoimmune phenomena and under the effects of immunosuppressive therapy. Moreover, the graft targeted by the immune reaction was not self but coming from a donor with a completely different genetic background, given the fact that selection was very uncommon, even for the HLA antigens. Soon after the first description, some authors started using other terms such as "*de novo* hepatitis"[3], "graft dysfunction mimicking autoimmune hepatitis"[16], "posttransplant immune hepatitis"[4] or *de novo* immune
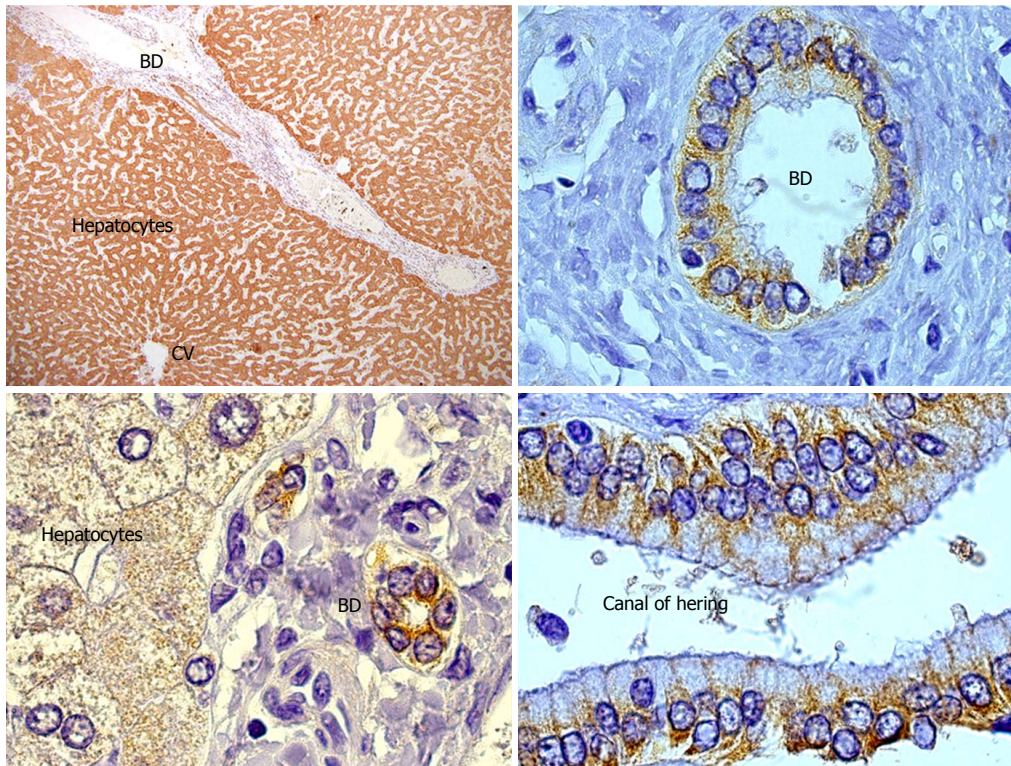
Figure 1 GSTT1 expression in human liver. On the left part of the figure the hepatic parenchyma shows homogeneous staining of the cytoplasm of hepatocytes as well as epithelial cells of the bile ducts in the portal area. On the right part, a detail of the cytoplasmic staining of cholangiocytes and the Canal of Hering cells, considered a niche of hepatic progenitor cells. CV: Central vein; BD: Bile duct.

hepatitis[18]. In 2008, a study performed by Fiel *et al*[23] introduced the term "plasma cell hepatitis" with the suggestion that dnAIH could be a variant of rejection; this report was highlighted by an editorial comment[34]. Even though all these terms have been used historically, for simplicity, in this review we will refer to dnAIH or PC-rich rejection.

GLUTATHIONE S-TRANSFERASE T1: A MINOR HISTOCOMPATIBILITY ANTIGEN

GSTT1 is a phase II drug metabolizing enzyme involved in protection against toxic compounds. Vital for this function is its abundant expression in liver and kidney (Figure 1), although GSTT1 is also expressed in other tissues to a lesser degree. Importantly, the GSTT1 antigen is present in red blood cells. It has been demonstrated that any healthy person with the *GSTT1*0/0* genotype might produce anti-GSTT1 antibodies if they experience one of two sensitization events: blood transfusions from a GSTT1-positive donor or pregnancy of a GSTT1-positive fetus[41]. Mismatch of the GSTT1 alleles was first reported by Aguilera *et al*[42] and subsequently confirmed in patients with PC-rich rejection[18,21,24,43]. Czaja included GSTT1 antibodies as a feature in dnAIH patients and proposed a hypothesis that will be discussed in the PATHOGENESIS OF PC-RICH REJECTION section of this manuscript[44]. Controversially, one report did not find GSTT1 mismatch

in a patient with dnAIH; however, the genotyping results were not very convincing since they lacked an internal control band and GSTT1 antibodies were not tested[22]. To the best of our knowledge, anti-GSTT1 antibodies have never been tested in serum samples from liver transplanted children.

We studied the cases of two female patients with preformed anti-GSTT1 antibodies who needed a liver transplant and received a GSTT1 expressing graft. Their original diseases were primary and secondary biliary cirrhosis, respectively. The first patient presented high titers of GSTT1 antibodies and was diagnosed with dnAIH 12 mo after the transplant with a good response to prednisone plus mycophenolate mofetil. She died of pneumonia 4 years after the transplant. The second patient had low antibody titers in different samples until exitus, 7 years after the transplant. This patient was never diagnosed correctly and the only biopsy, performed one month before exitus, revealed dnAIH as the cause of exitus. Since these pre-sensitized patients are not frequent, it is difficult to conclude whether the presence of preformed antibodies accelerates the onset or the severity of the disease.

IN FAVOR OF ANTIBODY-MEDIATED REJECTION IN *DE NOVO* AIH

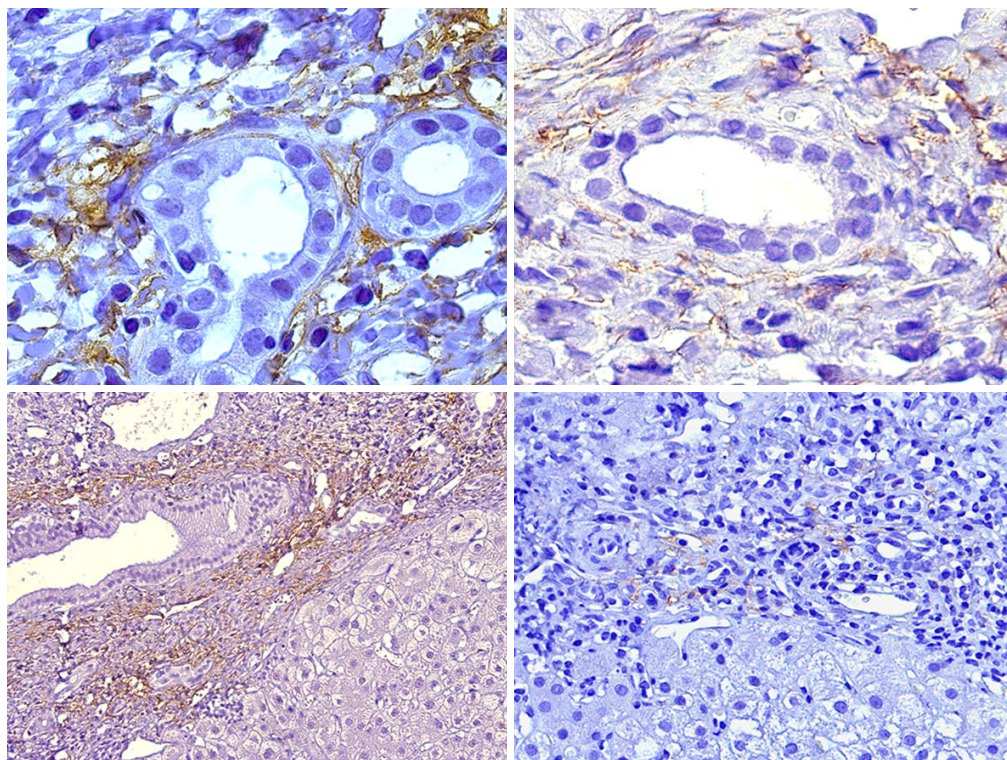At the beginning of this decade a report by Aguilera *et al*[36], that was worth an editorial comment[45] was

Figure 2 Portal areas in diagnostic biopsies of patients with plasma cell-rich rejection are shown. Staining of C4d deposits is observed in portal capillaries indicating antibody-mediated rejection.

the first to link C4d immunopositivity in liver biopsies to donor-specific alloreactivity not related to HLA DSA or ABO incompatibility[46]. Patients with anti-GSTT1 DSA showed a very characteristic C4d immunostaining pattern restricted to portal capillaries (Figure 2), very distinct to the sinusoidal pattern described by Kozlowski *et al*[47] in association with HLA DSA, which was similar to our own findings in chronic rejection biopsies[36].

Dumortier *et al*[48] described a case of refractory dnAIH that did not respond to standard therapy in a patient with anti-LKM (liver-kidney microsomal) antibodies at the time of diagnosis. Recovery after treatment with plasmapheresis supported an involvement of humoral factors in the pathogenesis of the disease.

In the last publication by the Banff Working Group for liver allograft pathology, the proposed criteria included HLA DSA and GSTT1 in null recipients of GSTT1⁻ positive donors as contributory but nonessential features for the diagnosis of PC-rich rejection[1].

HLA DQ DSAs have been described as a predictive variable for late allograft dysfunction in children[49]. Unfortunately, the number of patients in this study with dnAIH (*n* = 3) was too low to reach a conclusion and, even with the inclusion of 10 more patients with overlapping features of dnAIH and late ACR, the results should be considered carefully since it is not possible to analyze both events separately. We studied HLA DSA in a group of adult patients with dnAIH and found that 4 out of 14 (28.6%) produced *de novo* HLA DQ DSA, but always coexisting with GSTT1 DSAs (unpublished

results), making it impossible to differentiate the real impact of HLA DSA on the development of PC-rich rejection. It would be very interesting to validate these results in larger cohorts in order to determine the pathogenic role of HLA DSA in the absence of anti-GSTT1 antibodies.

THE THIN LINE BETWEEN AUTO- AND ALLO-IMMUNITY

Although finally accepted as a form of allograft rejection, the liver transplant-associated disease formerly known as dnAIH is a special kind of rejection and one that is difficult to understand. It has many histological similarities with classical AIH, features that were misleading at the time this pathology was discovered. Moreover, the first-line therapy of choice is corticosteroids for both pathological processes. All of this supports the concept that an alloantigenic immune response may be difficult to distinguish from an autoimmune response in which the mechanisms of liver damage are probably similar. Interestingly, we noted a female predominance among patients who present with one autoimmune feature, since of the cases of dnAIH diagnosed at our center, 10 of 14 are female (71.4%), a feature that is not easy to explain if we contemplate that 75% of the liver transplanted patients in our hospital are males. Moreover, if we consider the 122 patients within the risk group with GSTT1 mismatch (positive donor/null recipient), 85 were males (69.7%) and 37 were females (30.3%). In

Table 1 Reported cases of de novo autoimmune hepatitis in pediatric and adult liver transplantation				
Ref.	*de novo* AIH/total patients	Original disease (immune-mediated)	Initial calcineurine inhibitor	Pediatric/adult
Kerkar *et al*[2] (1998)	7/180	4 BA	4 CyA, 3 Tac	P
Gupta *et al*[3] (2001)	6/115	5 BA	6 CyA	P
Andries *et al*[4] (2001)	11/471	7 BA	10 Cya, 1 Tac	P
Hernández *et al*[5] (2001)	5/155	4 BA, 1 PSC	5 CyA	P
Petz *et al*[6] (2002)	18/155	16 BA	9 Cya, 9 Tac	P
Miyagawa-Hayashino *et al*[7] (2003)	1	BA	Tac	P
Gibelli *et al*[8] (2006)	2/206	1 BA	CyA	P
Evans *et al*[9] (2006)	4/158	4 PSC	4 CyA	P
Riva *et al*[10] (2006)	9/247	9 BA	5 CyA, 4 Tac	P
Oya *et al*[11] (2009)	1	-	Tac	P
Cho *et al*[12] (2011)	4/148	1 BA	-	P
Pongpaibul *et al*[13] (2012)	51/685	29 BA	22 CyA, 29 Tac	P
Ekong *et al*[14] (2017)	29/1833	17 BA	13 CyA,16 Tac	P
Jones *et al*[15] (1999)	2	2 PBC	2 CyA	A
Heneghan *et al*[16] (2001)	7/1000	1 PBC, 2 PSC	7 CyA	A
Salcedo *et al*[17] (2002)	12/350	1 BA 1 PSC	12 CyA	A
Aguilera *et al*[18] (2004)	6/110	None	5 CyA, 1 Tac	A
¹Update 2017	8	1 PBC, 1 PSC	8 CyA	A
Miyagawa-Hayashino *et al*[19] (2004)	13/633	11 BA	13 Tac	A
Tsuji *et al*[20] (2005)	1	PBC	CyA	A
Rodriguez-Diaz *et al*[21] (2006)	1	PBC	CyA	A
Yoshizawa *et al*[22] (2008)	1	PBC	Tac	A
Fiel *et al*[23] (2008)	38/?	none (HCV)	11 CyA, 27 Tac	A
Zhang *et al*[24] (2010)	1	PBC	Tac	A
Montano-Loza *et al*[25] (2012)	17/576	5 PBC, 1 BA	5 CyA, 7 Tac	A

¹Presently we have 8 more patients diagnosed. BA: Biliary atresia; PBC: Primary biliary cirrhosis; PSC: Primary sclerosing cholangitis; CyA: Cyclosporine A; Tac: Tacrolimus; A: Adult; P: Pediatric.

accordance with female predominance are the reports by Miyagawa-Hayashino[7,19], in which 12 of 14 cases were females, and Pongpaibul *et al*[13] with 32 females *vs* 19 males diagnosed with dnAIH, although in the majority of publications this predominance does not appear. Ward *et al*[50] established that women may be more susceptible to PC hepatitis after liver transplant than men. A reasonable explanation could be sensitization through pregnancies. In fact, we have two female patients with GSTT1 antibodies in pre-transplant serum samples, although we do not have a sufficient number of cases to support this hypothesis.

AUTOIMMUNE OR IMMUNE MEDIATED PRETRANSPLANT PATHOLOGIES: HAVE THEY ANY INFLUENCE IN PC-RICH REJECTION?

It is generally accepted that dnAIH develops in patients transplanted for non-autoimmune diseases. This is not exactly true since there are a number of patients whose original disease was either primary biliary cirrhosis (PBC) or primary sclerosing cholangitis (PSC) (Table 1). Neuberger's group found that PBC was an independent predictor of late acute rejection in a large cohort of adult liver transplant patients[51].

In children, the major indication for liver transplant is

biliary atresia (BA), a process of unknown etiology with several potential causative factors. Among them, there is evidence in favor of autoimmune-mediated injury of bile duct epithelial cells in a subset of patients[52-54]. Very recently, a study described that BA is initiated before birth and the presence of maternal microchimerism in the BA liver supports graft versus host disease (GvHD)-like immune response[55].

T CELL INVOLVEMENT IN PC-RICH REJECTION

A possible role of GSTT1-specific T lymphocytes in PC-rich rejection has been recently investigated. The authors provided the first evidence of memory specific T cells in samples of peripheral blood mononuclear cells (PBMC) of patients after recall with the GSTT1 antigen. Activation of CD8^{+} and CD4^{+} T cells with production of IL-4 and/or IFNγ was observed[56]. Most intriguing was the finding of highly activated CD4^{+} cells that retained CD8^{low} expression with a mean value of 25% activated Th0 type cells (3.44%-78.95%). These results are particularly significant considering the immunosuppressed status of the patients. In silico analysis of the ability of patients' HLA class I and class II alleles to present these peptides with optimal percentile ranks supported the experimental results[56].
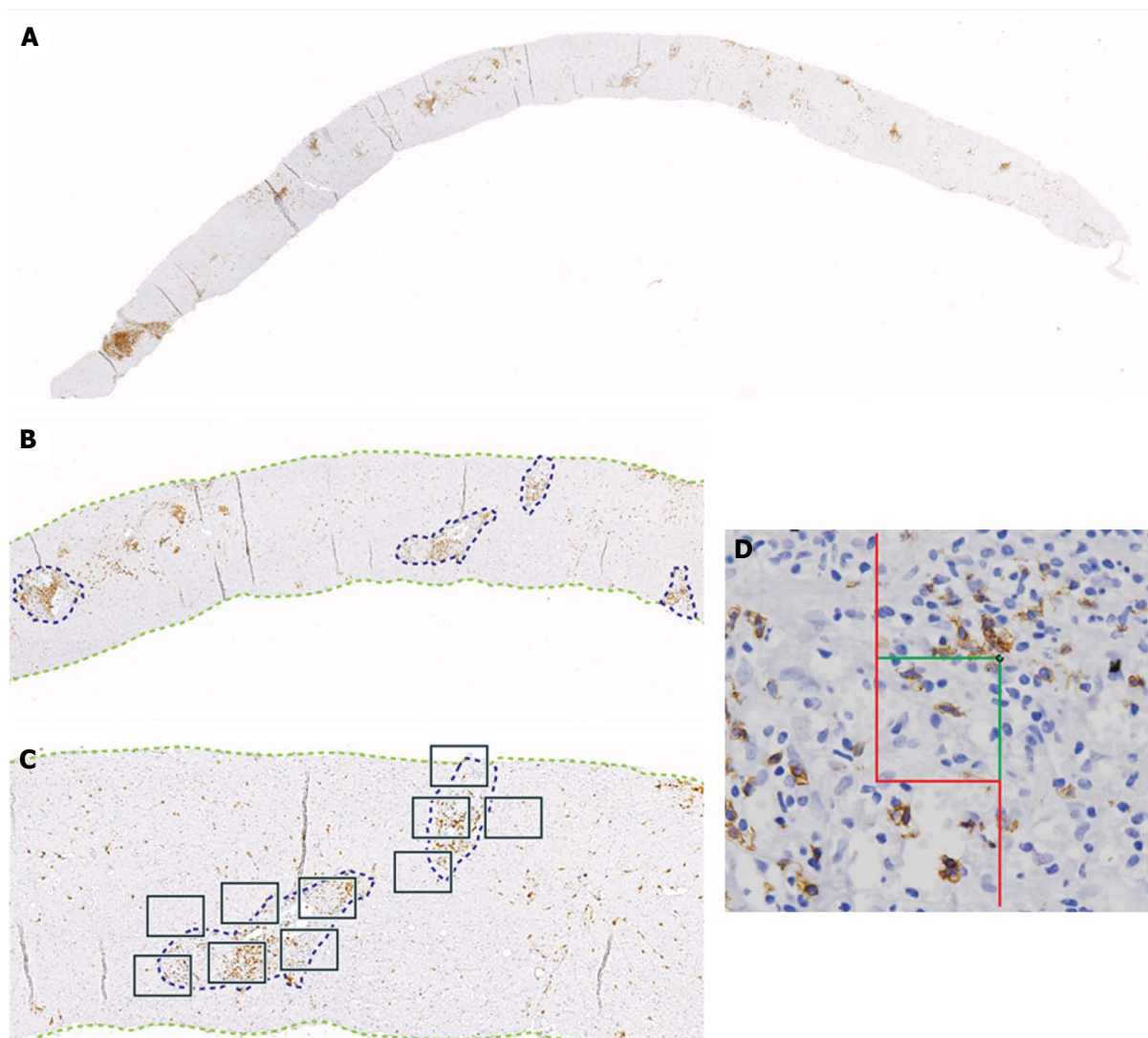
Similar to autoimmune hepatitis, we observe damage

Figure 3 Representative images showing quantification procedure using NewCAST™ Visiopharm® software. A: Super image capture of a slide; B: Computer-assisted drawing of the tissue (green) and manually drawing of the regions of interest (ROI) or portal areas (blue); C: Example of the unbiased sampling; D: A detail of cell quantification inside the counting frame.

to hepatocytes that is believed to be coordinated by CD4^{+} T lymphocytes recognizing a self-antigen presented by HLA-class II molecules to Th0 type cells[57], although with the substantial difference that, in this case, the target is an alloantigen.

WHICH CELLS ARE INFILTRATING THE LIVER AT THE ONSET OF PC-RICH REJECTION?

Diagnosis by liver biopsy is challenging because PC-rich rejection shares some histological and clinical features with late onset acute rejection or with other post-transplant pathologies such as recurrent hepatitis C. Moreover, very little is known about the cellular composition of the inflammatory infiltrates in portal tracts other than an important presence of plasma cells. Direct visualization of the cell types with different markers and quantification of cells/mm^2 of tissue in diagnostic biopsies using computer-assisted system technology (newCAST) has proved to be an unbiased way to evaluate the situation directly in the organ and not only in peripheral blood (Figure 3). Very recent data allowed us to define a cellular profile characteristic of PC-rich rejection at the time of diagnosis[58]. We observed a predominance of T lymphocytes (mean 36.6%); followed by PCs (mean 28.8%), with 17% of them IgG4^{+}; B cells (mean 14.9%); and macrophages (mean 19.7%) (Figure 4). This profile was very different to the one defined in a chronic rejection group included in the same study[58]. It remains to be confirmed whether the cellular composition in acute rejection biopsies would be useful to support diagnosis since HCV recurrence no longer has the same impact in the transplant community that as in the past.

The IgG4 subclass has traditionally been considered a modulator of the immune response, linked to long-
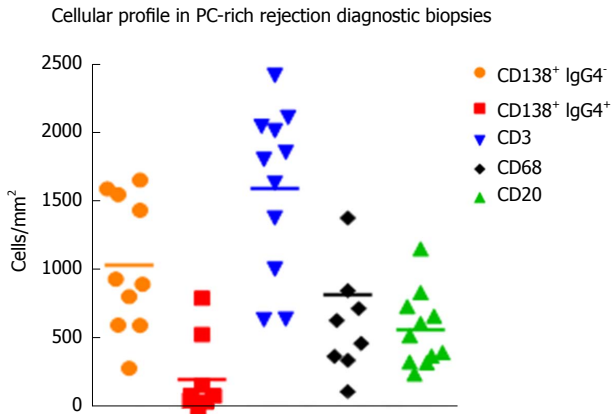
Cellular profile in PC-rich rejection diagnostic biopsies

Figure 4 Total cell count/ area of tissuein diagnostic biopsies with the following markers: CD138+ plasma cells, IgG4+ plasma cells, CD3+ T lymphocytes, CD68+ macrophages, CD20+ B lymphocytes. Mean values are shown.

term exposure and high antigen concentration. Some authors analyzed the presence of IgG4 antibodies in dnAIH, mostly in children, but the results were contradictory. While Eguchi *et al*[59] described the absence of IgG4+ plasma cells in the biopsies of 4 patients and the values in serum were within normal limits, Castillo-Rama *et al*[60] detected IgG4+ PC over-representation in a subgroup of patients with PC hepatitis that showed a more severe disease course. The origin of these IgG4+ PC infiltrates in the allografts is unknown but it could be due to persistent alloimmune responses. We also found an important number of IgG4+ PC, representing 16.48% (2.45%-65%) of the total PCs in diagnostic biopsies of patients with PC-rich rejection (Figure 5A and B); however, higher numbers of IgG4+cells was not associated with worse clinical outcome. We also found an over-representation of serum levels of GSTT1-specific IgG4 antibodies, almost equaling the levels of GSTT1-IgG1, while GSTT1-IgG2 and -IgG3 were completely absent (Figure 5C)[61].

There is very little information about the representation of T cells in PC-rich rejection biopsies. A study performed by Ekong *et al*[62] found that around 3000 CD3+ cells/mm2 were present in the dnAIH pediatric group during disease activity. Our data in adults showed that CD3+ cells were less numerous, with a mean value of 1600 (641-2422) cells/mm2 at the time of diagnosis, before steroid treatment. Indirect evidence from biopsies suggests a potential role for Th17 cells in the prolongation of inflammation in dnAIH[63] although this needs further study.

PC-RICH REJECTION IN HEMATOPOIETIC CELL TRANSPLANTATION

PC-rich rejection has also been identified after hematopoietic cell transplantation (HCT) between HLA-identical siblings. In this context, the GSTT1 mismatch, defined as null donor/positive recipient, not only had a

deleterious effect in HCT and constituted a risk factor for acute and chronic hepatic GvHD[64] but is also the basis of a true PC-rich rejection[65]. Together with the skin and intestine, the liver is a preferred target of the donor cells which are able to recognize disparate antigens expressed by the recipient. Differential diagnosis of hepatic GvHD versus PC-rich rejection must be done in biopsy because histological features can only be analyzed in tissue to permit the differentiation of both processes. As it happens, the cellular composition of the inflammatory infiltrates in portal areas of HCT patient with PC-rich rejection was very similar to that of diagnostic biopsies of liver transplanted patients[58].

From an immunological point of view, this finding has important implications because it demonstrates an identical immune response against the GSTT1 antigen as a result of donor/recipient mismatch in two completely different conditions: Solid organ and HCT.

PATHOGENESIS OF PC-RICH REJECTION

It is clear that during surgery, intracellular antigens such as the GSTT1 protein are released and recognized by B cells, starting a process of affinity maturation and differentiation into memory cells, which requires collaboration with specific T helper cells in the lymph nodes. If this occurs, a subset of these cells will progress to plasma cells and anti-GSTT1 antibodies of the IgG class will be detected concurrently with mature GSTT1-specific CD4+ cells. Our experimental results sustain indirect presentation of the donor GSTT1 protein by recipient APCs[56].

In general terms, we could use the model proposed by Czaja[44] with some suggestions. Briefly, inflammatory stimuli induce expression of MHC class II in hepatocytes and cholangiocytes and, since both cellular types contain abundant GSTT1 enzyme, these liver cells could directly act as APCs and serve as targets of effector CD4+ T cells, whose existence has been also demonstrated in the PBMC of patients with the disease. The presence of an antigen mimicking GSTT1 on the surface of the cell cannot be ruled out but it does not seem necessary and so far has not been identified.

GSTT1-specific B cells have a critical role as antigen presenting cells (APCs) in the maintenance of a long-lasting immunological response, providing the signals required for specific T cell activation when professional APCs become exhausted. GSTT1 antibodies are, in principle and until a direct pathogenic role can be demonstrated, a signal of the existence of memory B cells. The mechanisms controlling GSTT1-specific T cell response in patients that, in spite of the production of GSTT1 antibodies, do not develop the disease are still not known. Alternatively, there are patients with GSTT1-specific T cells that lack the B cell response; they will never develop PC-rich rejection. In our experience, if PC-rich rejection is not initiated during the first 3 years, it will never occur even though anti-
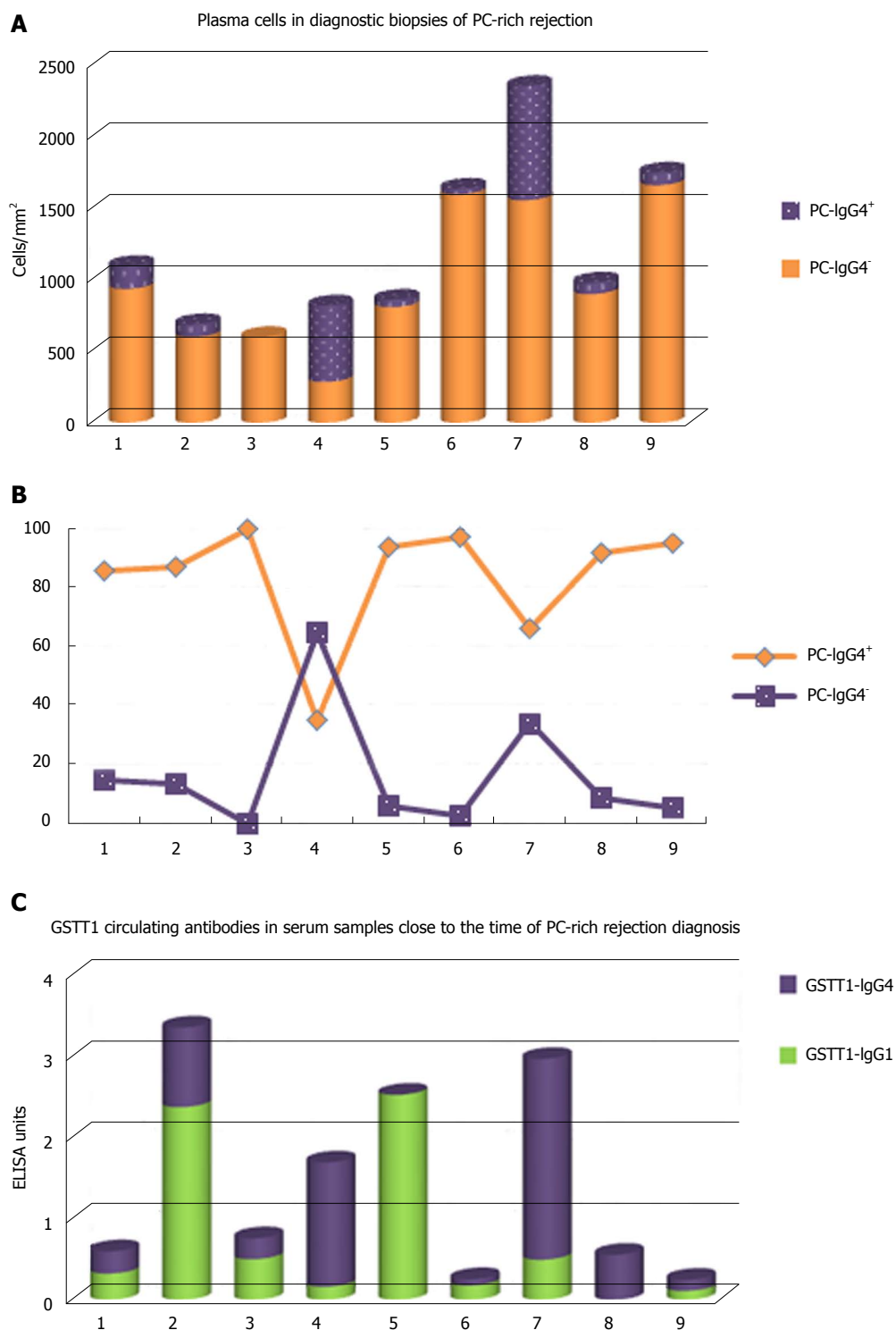
A

Plasma cells in diagnostic biopsies of PC-rich rejection

B

C

GSTT1 circulating antibodies in serum samples close to the time of PC-rich rejection diagnosis

Figure 5 CD138^{+} plasma cells were quantified in the diagnostic biopsies of the 7 patients (A). The results are shown as number of cells per mm²/tissue. IgG4^{+} plasma cells were also counted and subtracted from the total number of CD138^{+} cells. B: The same results are represented as percentages. C: Level of anti-GSTT1 antibodies in serum samples close to the diagnostic biopsy of 7 patients with dnAIH; note that only IgG1 and IgG4 were present whereas IgG2 and IgG3 were absent. It is important to highlight that these are donor-specific antibodies.

GSTT1 antibodies persist (longest follow-up > 20 years) (unpublished results).

Impairment of Treg cells has been described in patients with classical AIH[57]. In the transplant setting, a similar role for Tregs in the development of dnAIH has been suggested. Kerkar and Yanni proposed that Treg function could be impaired in dnAIH since calcineurin

inhibitors reduce the production of IL-2, which is required for the survival and proliferation of Tregs[66]. In the same sense, a study by Arterbery *et al*[67] sustains that the Tregs of patients with dnAIH are functionally impaired and produce increased levels of proinflammatory cytokines.

On the other hand, important questions about dnAIH

have yet to be answered. One of the most intriguing aspects is why some patients do not develop a GSTT1-specific B cell response, as this is going to be critical to the prevention of PC-rich rejection. In our hands, the choice of tacrolimus instead of cyclosporine can be decisive in avoiding the humoral response[68]. However, there is no consensus among the scientific community on this matter. While some studies support the observation of tacrolimus as a protective factor[3-5,8,9,15-17,20,21], others have shown that the use of tacrolimus seems to be a risk factor for dnAIH[13,14,19,22-25]; therefore, this point remains controversial (Table 1).

CONCLUSION

We are now closer than ever to clarification of the mechanisms leading to plasma cell-rich rejection. The demonstration of memory T cells specific for the GSTT1 antigen as well as B lymphocytes and GSTT1 antibody-producing plasma cells after GSTT1-mismatched liver transplants support the hypothesis in which both cell types are required to develop the immune response leading to PC-rich rejection. This chronic disease has a significant impact on survival of those patients that are not correctly diagnosed.

REFERENCES

1   **Demetris AJ**, Bellamy C, Hübscher SG, O'Leary J, Randhawa PS, Feng S, Neil D, Colvin RB, McCaughan G, Fung JJ, Del Bello A, Reinholt FP, Haga H, Adeyi O, Czaja AJ, Schiano T, Fiel MI, Smith ML, Sebagh M, Tanigawa RY, Yilmaz F, Alexander G, Baiocchi L, Balasubramanian M, Batal I, Bhan AK, Bucuvalas J, Cerski CTS, Charlotte F, de Vera ME, ElMonayeri M, Fontes P, Furth EE, Gouw ASH, Hafezi-Bakhtiari S, Hart J, Honsova E, Ismail W, Itoh T, Jhala NC, Khettry U, Klintmalm GB, Knechtle S, Koshiba T, Kozlowski T, Lassman CR, Lerut J, Levitsky J, Licini L, Liotta R, Mazariegos G, Minervini MI, Misdraji J, Mohanakumar T, Mölne J, Nasser I, Neuberger J, O'Neil M, Pappo O, Petrovic L, Ruiz P, Sağol Ö, Sanchez Fueyo A, Sasatomi E, Shaked A, Shiller M, Shimizu T, Sis B, Sonzogni A, Stevenson HL, Thung SN, Tisone G, Tsamandas AC, Wernerson A, Wu T, Zeevi A, Zen Y. 2016 Comprehensive Update of the Banff Working Group on Liver Allograft Pathology: Introduction of Antibody-Mediated Rejection. *Am J Transplant* 2016; **16**: 2816-2835 [PMID: 27273869 DOI: 10.1111/ajt.13909]
2   **Kerkar N**, Hadzić N, Davies ET, Portmann B, Donaldson PT, Rela M, Heaton ND, Vergani D, Mieli-Vergani G. De-novo autoimmune hepatitis after liver transplantation. *Lancet* 1998; **351**: 409-413 [PMID: 9482295 DOI: 10.1016/S0140-6736(97)06478-7]
3   **Gupta P**, Hart J, Millis JM, Cronin D, Brady L. De novo hepatitis with autoimmune antibodies and atypical histology: a rare cause of late graft dysfunction after pediatric liver transplantation. *Transplantation* 2001; **71**: 664-668 [PMID: 11292299]
4   **Andries S**, Casamayou L, Sempoux C, Burlet M, Reding R, Bernard Otte J, Buts JP, Sokal E. Posttransplant immune hepatitis in pediatric liver transplant recipients: incidence and maintenance therapy with azathioprine. *Transplantation* 2001; **72**: 267-272 [PMID: 11477351]
5   **Hernandez HM**, Kovarik P, Whitington PF, Alonso EM. Autoimmune hepatitis as a late complication of liver transplantation. *J Pediatr Gastroenterol Nutr* 2001; **32**: 131-136 [PMID: 11321380]
6   **Petz W**, Sonzogni A, Bertani A, Spada M, Lucianetti A, Colledan M, Gridelli B. A cause of late graft dysfunction after pediatric liver transplantation: de novo autoimmune hepatitis. *Transplant*

Proc 2002; **34**: 1958-1959 [PMID: 12176643 DOI: 10.1016/S0041-1345(02)03137-8]
7   **Miyagawa-Hayashino A**, Haga H, Sakurai T, Shirase T, Manabe T, Egawa H. De novo autoimmune hepatitis affecting allograft but not the native liver in auxiliary partial orthotopic liver transplantation. *Transplantation* 2003; **76**: 271-272 [PMID: 12865828 DOI: 10.1097/01.TP.0000072338.88465.59]
8   **Gibelli NE**, Tannuri U, Mello ES, Cançado ER, Santos MM, Ayoub AA, Maksoud-Filho JG, Velhote MC, Silva MM, Pinho-Apezzato ML, Maksoud JG. Successful treatment of de novo autoimmune hepatitis and cirrhosis after pediatric liver transplantation. *Pediatr Transplant* 2006; **10**: 371-376 [PMID: 16677364 DOI: 10.1111/j.1399-3046.2005.00470.x]
9   **Evans HM**, Kelly DA, McKiernan PJ, Hübscher S. Progressive histological damage in liver allografts following pediatric liver transplantation. *Hepatology* 2006; **43**: 1109-1117 [PMID: 16628633 DOI: 10.1002/hep.21152]
10   **Riva S**, Sonzogni A, Bravi M, Bertani A, Alessio MG, Candusso M, Stroppa P, Melzi ML, Spada M, Gridelli B, Colledan M, Torre G. Late graft dysfunction and autoantibodies after liver transplantation in children: preliminary results of an Italian experience. *Liver Transpl* 2006; **12**: 573-577 [PMID: 16555335 DOI: 10.1002/lt.20673]
11   **Oya H**, Sato Y, Yamamoto S, Kobayashi T, Watanabe T, Kokai H, Hatakeyama K. De novo autoimmune hepatitis after living donor liver transplantation in a 25-day-old newborn baby: a case report. *Transplant Proc* 2009; **41**: 433-434 [PMID: 19249573 DOI: 10.1016/j.transproceed.2008.10.031]
12   **Cho JM**, Kim KM, Oh SH, Lee YJ, Rhee KW, Yu E. De novo autoimmune hepatitis in Korean children after liver transplantation: a single institution's experience. *Transplant Proc* 2011; **43**: 2394-2396 [PMID: 21839275 DOI: 10.1016/j.transproceed.2011.05.030]
13   **Pongpaibul A**, Venick RS, McDiarmid SV, Lassman CR. Histopathology of de novo autoimmune hepatitis. *Liver Transpl* 2012; **18**: 811-818 [PMID: 22378542 DOI: 10.1002/lt.23422]
14   **Ekong UD**, McKiernan P, Martinez M, Lobritto S, Kelly D, Ng VL, Alonso EM, Avitzur Y. Long-term outcomes of de novo autoimmune hepatitis in pediatric liver transplant recipients. *Pediatr Transplant* 2017; **21** [PMID: 28556542 DOI: 10.1111/petr.12945]
15   **Jones DE**, James OF, Portmann B, Burt AD, Williams R, Hudson M. Development of autoimmune hepatitis following liver transplantation for primary biliary cirrhosis. *Hepatology* 1999; **30**: 53-57 [PMID: 10385638 DOI: 10.1002/hep.510300103]
16   **Heneghan MA**, Portmann BC, Norris SM, Williams R, Muiesan P, Rela M, Heaton ND, O'Grady JG. Graft dysfunction mimicking autoimmune hepatitis following liver transplantation in adults. *Hepatology* 2001; **34**: 464-470 [PMID: 11526530 DOI: 10.1053/jhep.2001.26756]
17   **Salcedo M**, Vaquero J, Bañares R, Rodríguez-Mahou M, Alvarez E, Vicario JL, Hernández-Albújar A, Tíscar JL, Rincón D, Alonso S, De Diego A, Clemente G. Response to steroids in de novo autoimmune hepatitis after liver transplantation. *Hepatology* 2002; **35**: 349-356 [PMID: 11826408 DOI: 10.1053/jhep.2002.31167]
18   **Aguilera I**, Sousa JM, Gavilán F, Bernardos A, Wichmann I, Nuñez-Roldán A. Glutathione S-transferase T1 mismatch constitutes a risk factor for de novo immune hepatitis after liver transplantation. *Liver Transpl* 2004; **10**: 1166-1172 [PMID: 15350010 DOI: 10.1002/lt.20209]
19   **Miyagawa-Hayashino A**, Haga H, Egawa H, Hayashino Y, Sakurai T, Minamiguchi S, Tanaka K, Manabe T. Outcome and risk factors of de novo autoimmune hepatitis in living-donor liver transplantation. *Transplantation* 2004; **78**: 128-135 [PMID: 15257051]
20   **Tsuji H**, Hiramatsu K, Minato H, Kaneko S, Nakanuma Y. Auxiliary partial orthotopic liver transplantation with de novo autoimmune hepatitis in the allograft and leftover primary biliary cirrhosis in the native liver. *Semin Liver Dis* 2005; **25**: 371-377 [PMID: 16143952 DOI: 10.1055/s-2005-916328]
21   **Rodríguez-Diaz Y**, Reyes-Rodriguez R, Dorta-Francisco MC,

Aguilera I, Perera-Molinero A, Moneva-Arce E, Aviles-Ruiz JF. De novo autoimmune hepatitis following liver transplantation for primary biliary cirrhosis. *Transplant Proc* 2006; **38**: 1467-1470 [PMID: 16797335 DOI: 10.1016/j.transproceed.2006.03.071]

22   **Yoshizawa K**, Shirakawa H, Ichijo T, Umemura T, Tanaka E, Kiyosawa K, Imagawa E, Matsuda K, Hidaka E, Sano K, Nakazawa Y, Ikegami T, Hashikura Y, Miyagawa S, Ota M, Nakano M. De novo autoimmune hepatitis following living-donor liver transplantation for primary biliary cirrhosis. *Clin Transplant* 2008; **22**: 385-390 [PMID: 18190552 DOI: 10.1111/j.1399-0012.2007.00787.x]

23   **Fiel MI**, Agarwal K, Stanca C, Elhajj N, Kontorinis N, Thung SN, Schiano TD. Posttransplant plasma cell hepatitis (de novo autoimmune hepatitis) is a variant of rejection and may lead to a negative outcome in patients with hepatitis C virus. *Liver Transpl* 2008; **14**: 861-871 [PMID: 18508382 DOI: 10.1002/lt.21447]

24   **Zhang Y**, Wang B, Wang T. De novo autoimmune hepatitis with centrilobular necrosis following liver transplantation for primary biliary cirrhosis: a case report. *Transplant Proc* 2010; **42**: 3854-3857 [PMID: 21094869 DOI: 10.1016/j.transproceed.2010.08.062]

25   **Montano-Loza AJ**, Vargas-Vorackova F, Ma M, Bain VG, Burak K, Kumar T, Mason AL. Incidence and risk factors associated with de novo autoimmune hepatitis after liver transplantation. *Liver Int* 2012; **32**: 1426-1433 [PMID: 22712495 DOI: 10.1111/j.1478-3231.2012.02832.x]

26   **Banff Working Group.**, Demetris AJ, Adeyi O, Bellamy CO, Clouston A, Charlotte F, Czaja A, Daskal I, El-Monayeri MS, Fontes P, Fung J, Gridelli B, Guido M, Haga H, Hart J, Honsova E, Hubscher S, Itoh T, Jhala N, Jungmann P, Khettry U, Lassman C, Ligato S, Lunz JG 3rd, Marcos A, Minervini MI, Mölne J, Nalesnik M, Nasser I, Neil D, Ochoa E, Pappo O, Randhawa P, Reinholt FP, Ruiz P, Sebagh M, Spada M, Sonzogni A, Tsamandas AC, Wernerson A, Wu T, Yilmaz F. Liver biopsy interpretation for causes of late liver allograft dysfunction. *Hepatology* 2006; **44**: 489-501 [PMID: 16871565 DOI: 10.1002/hep.21280]

27   **Shaikh OS**, Demetris AJ. Idiopathic posttransplantation hepatitis? *Liver Transpl* 2007; **13**: 943-946 [PMID: 17600346 DOI: 10.1002/lt.21202]

28   **Schreuder TC**, Hübscher SG, Neuberger J. Autoimmune liver diseases and recurrence after orthotopic liver transplantation: what have we learned so far? *Transpl Int* 2009; **22**: 144-152 [PMID: 18662365 DOI: 10.1111/j.1432-2277.2008.00729.x]

29   **Hübscher SG**. Antibody-mediated rejection in the liver allograft. *Curr Opin Organ Transplant* 2012; **17**: 280-286 [PMID: 22569512 DOI: 10.1097/MOT.0b013e328353584c]

30   **Hübscher SG**. What is the long-term outcome of the liver allograft? *J Hepatol* 2011; **55**: 702-717 [PMID: 21426919 DOI: 10.1016/j.jhep.2011.03.005]

31   **Sebagh M**, Castillo-Rama M, Azoulay D, Coilly A, Delvart V, Allard MA, Dos Santos A, Johanet C, Roque-Afonso AM, Saliba F, Duclos-Vallée JC, Samuel D, Demetris AJ. Histologic findings predictive of a diagnosis of de novo autoimmune hepatitis after liver transplantation in adults. *Transplantation* 2013; **96**: 670-678 [PMID: 23982338 DOI: 10.1097/TP.0b013e31829eda7f]

32   **Vukotic R**, Vitale G, D'Errico-Grigioni A, Muratori L, Andreone P. De novo autoimmune hepatitis in liver transplant: State-of-the-art review. *World J Gastroenterol* 2016; **22**: 2906-2914 [PMID: 26973387 DOI: 10.3748/wjg.v22.i10.2906]

33   **Nagai S**, Ito M, Kamei H, Nakamura T, Ando H, Kiuchi T. Indirect immunohistochemical evaluation of graft fibrosis and interface hepatitis after pediatric liver transplantation. *Pediatr Transplant* 2010; **14**: 342-350 [PMID: 19744282 DOI: 10.1111/j.1399-3046.2009.01234.x]

34   **Demetris AJ**, Sebagh M. Plasma cell hepatitis in liver allografts: Variant of rejection or autoimmune hepatitis? *Liver Transpl* 2008; **14**: 750-755 [PMID: 18508366 DOI: 10.1002/lt.21518]

35   **Solez K**, Colvin RB, Racusen LC, Haas M, Sis B, Mengel M, Halloran PF, Baldwin W, Banfi G, Collins AB, Cosio F, David DS, Drachenberg C, Einecke G, Fogo AB, Gibson IW, Glotz D, Iskandar SS, Kraus E, Lerut E, Mannon RB, Mihatsch M, Nankivell BJ, Nickeleit V, Papadimitriou JC, Randhawa P, Regele H, Renaudin K, Roberts I, Seron D, Smith RN, Valente M. Banff 07 classification of renal allograft pathology: updates and future directions. *Am J Transplant* 2008; **8**: 753-760 [PMID: 18294345 DOI: 10.1111/j.1600-6143.2008.02159.x]

36   **Aguilera I**, Sousa JM, Gavilan F, Gomez L, Alvarez-Márquez A, Núñez-Roldán A. Complement component 4d immunostaining in liver allografts of patients with de novo immune hepatitis. *Liver Transpl* 2011; **17**: 779-788 [PMID: 21425430 DOI: 10.1002/lt.22302]

37   **Trivedi A**, Schiano TD, Ward SC, Thung SN, Fiel MI, Levitsky J. C4d is present in portal venules in plasma cell hepatitis and may also be used as a predictor for its development. *Hepatology* 2014; **60**: 457A

38   **Deshpande V**, Zen Y, Chan JK, Yi EE, Sato Y, Yoshino T, Klöppel G, Heathcote JG, Khosroshahi A, Ferry JA, Aalberse RC, Bloch DB, Brugge WR, Bateman AC, Carruthers MN, Chari ST, Cheuk W, Cornell LD, Fernandez-Del Castillo C, Forcione DG, Hamilos DL, Kamisawa T, Kasashima S, Kawa S, Kawano M, Lauwers GY, Masaki Y, Nakanuma Y, Notohara K, Okazaki K, Ryu JK, Saeki T, Sahani DV, Smyrk TC, Stone JR, Takahira M, Webster GJ, Yamamoto M, Zamboni G, Umehara H, Stone JH. Consensus statement on the pathology of IgG4-related disease. *Mod Pathol* 2012; **25**: 1181-1192 [PMID: 22596100 DOI: 10.1038/modpathol.2012.72]

39   **Tamaro G**, Sonzogni A, Torre G. Monitoring "de novo" autoimmune hepatitis (LKM positive) by serum type-IV collagen after liver transplant: a paediatric case. *Clin Chim Acta* 2001; **310**: 25-29 [PMID: 11485751 DOI: 10.1016/S0009-8981(01)00509-5]

40   **Tripathi D**, Neuberger J. Autoimmune hepatitis and liver transplantation: indications, results, and management of recurrent disease. *Semin Liver Dis* 2009; **29**: 286-296 [PMID: 19676001 DOI: 10.1055/s-0029-1233531]

41   **Wichmann I**, Aguilera I, Sousa JM, Bernardos A, García Núñez EJ, Vigil E, Magariño R, Magariño I, Torres A, Núñez-Roldán A. Antibodies against glutathione S-transferase T1 in non-solid organ transplanted patients. *Transfusion* 2006; **46**: 1505-1509 [PMID: 16965576 DOI: 10.1111/j.1537-2995.2006.00938.x]

42   **Aguilera I**, Wichmann I, Sousa JM, Bernardos A, Franco E, García-Lozano JR, Núñez-Roldán A. Antibodies against glutathione S-transferase T1 (GSTT1) in patients with de novo immune hepatitis following liver transplantation. *Clin Exp Immunol* 2001; **126**: 535-539 [PMID: 11737073]

43   **Rodriguez-Mahou M**, Salcedo M, Fernandez-Cruz E, Tiscar JL, Bañares R, Clemente G, Vicario JL, Alvarez E, Rodriguez-Sainz C. Antibodies against glutathione S-transferase T1 (GSTT1) in patients with GSTT1 null genotype as prognostic marker: long-term follow-up after liver transplantation. *Transplantation* 2007; **83**: 1126-1129 [PMID: 17452905 DOI: 10.1097/01.tp.0000259963.47350.da]

44   **Czaja AJ**. Diagnosis, pathogenesis, and treatment of autoimmune hepatitis after liver transplantation. *Dig Dis Sci* 2012; **57**: 2248-2266 [PMID: 22562533 DOI: 10.1007/s10620-012-2179-3]

45   **Bellamy CO**. Complement C4d immunohistochemistry in the assessment of liver allograft biopsy samples: applications and pitfalls. *Liver Transpl* 2011; **17**: 747-750 [PMID: 21542127]

46   **Haga H**, Egawa H, Fujimoto Y, Ueda M, Miyagawa-Hayashino A, Sakurai T, Okuno T, Koyanagi I, Takada Y, Manabe T. Acute humoral rejection and C4d immunostaining in ABO blood type-incompatible liver transplantation. *Liver Transpl* 2006; **12**: 457-464 [PMID: 16498648 DOI: 10.1002/lt.20652]

47   **Kozlowski T**, Rubinas T, Nickeleit V, Woosley J, Schmitz J, Collins D, Hayashi P, Passannante A, Andreoni K. Liver allograft antibody-mediated rejection with demonstration of sinusoidal C4d staining and circulating donor-specific antibodies. *Liver Transpl* 2011; **17**: 357-368 [PMID: 21445918 DOI: 10.1002/lt.22233]

48   **Dumortier J**, Scoazec JY, Guillaud O, Hequet O, Hervieu V, Boillot O. Treatment of severe refractory de novo auto-immune hepatitis after liver transplantation with plasmapheresis. *Clin Res Hepatol Gastroenterol* 2015; **39**: e83-e85 [PMID: 26070570 DOI: 10.1016/j.clinre.2015.04.003]

49 **Wozniak LJ**, Hickey MJ, Venick RS, Vargas JH, Farmer DG, Busuttil RW, McDiarmid SV, Reed EF. Donor-specific HLA Antibodies Are Associated With Late Allograft Dysfunction After Pediatric Liver Transplantation. *Transplantation* 2015; **99**: 1416-1422 [PMID: 26038872 DOI: 10.1097/TP.0000000000000796]

50 **Ward SC**, Schiano TD, Thung SN, Fiel MI. Plasma cell hepatitis in hepatitis C virus patients post-liver transplantation: case-control study showing poor outcome and predictive features in the liver explant. *Liver Transpl* 2009; **15**: 1826-1833 [PMID: 19938116 DOI: 10.1002/lt.21949]

51 **Neuberger J**. An update on liver transplantation: A critical review. *J Autoimmun* 2016; **66**: 51-59 [PMID: 26350881 DOI: 10.1016/j.jaut.2015.08.021]

52 **Mack CL**. What Causes Biliary Atresia? Unique Aspects of the Neonatal Immune System Provide Clues to Disease Pathogenesis. *Cell Mol Gastroenterol Hepatol* 2015; **1**: 267-274 [PMID: 26090510 DOI: 10.1016/j.jcmgh.2015.04.001]

53 **Alvarez F**. Is biliary atresia an immune mediated disease? *J Hepatol* 2013; **59**: 648-650 [PMID: 23792027 DOI: 10.1016/j.jhep.2013.06.006]

54 **Pang SY**, Dai YM, Zhang RZ, Chen YH, Peng XF, Fu J, Chen ZR, Liu YF, Yang LY, Wen Z, Yu JK, Liu HY. Autoimmune liver disease-related autoantibodies in patients with biliary atresia. *World J Gastroenterol* 2018; **24**: 387-396 [PMID: 29391761 DOI: 10.3748/wjg.v24.i3.387]

55 **Muraji T**, Ohtani H, Ieiri S. Unique manifestations of biliary atresia provide new immunological insight into its etiopathogenesis. *Pediatr Surg Int* 2017; **33**: 1249-1253 [PMID: 29022092 DOI: 10.1007/s00383-017-4155-7]

56 **Martínez-Bravo MJ**, Sánchez B, Sousa JM, Acevedo MJ, Gómez-Bravo MA, Núñez-Roldán A, Aguilera I. T-cell allorecognition of donor glutathione S-transferase T1 in plasma cell-rich rejection. *World J Hepatol* 2017; **9**: 1115-1124 [PMID: 29026463 DOI: 10.4254/wjh.v9.i27.1115]

57 **Vergani D**, Mieli-Vergani G. The impact of autoimmunity on hepatocytes. *Semin Liver Dis* 2007; **27**: 140-151 [PMID: 17520514 DOI: 10.1055/s-2007-979467]

58 **Aguado-Domínguez E**, Gómez L, Sousa JM, Gómez-Bravo MÁ, Núñez-Roldán A, Aguilera I. Identification of the cellular components involved in de novo immune hepatitis: a quantitative immunohistochemical analysis. *J Transl Med* 2018; **16**: 62 [PMID: 29534755 DOI: 10.1186/s12967-018-1440-8]

59 **Eguchi S**, Takatsuki M, Hidaka M, Tajima Y, Zen Y, Nakanuma Y, Kanematsu T. De novo autoimmune hepatitis after living donor liver transplantation is unlikely to be related to immunoglobulin subtype 4-related immune disease. *J Gastroenterol Hepatol* 2008; **23**: e165-e169 [PMID: 18505414 DOI: 10.1111/j.1440-1746.2008.05347.x]

60 **Castillo-Rama M**, Sebagh M, Sasatomi E, Randhawa P, Isse K, Salgarkar AD, Ruppert K, Humar A, Demetris AJ. "Plasma cell hepatitis" in liver allografts: identification and characterization of an IgG4-rich cohort. *Am J Transplant* 2013; **13**: 2966-2977 [PMID: 24011021 DOI: 10.1111/ajt.12413]

61 **Aguilera I**, Martinez-Bravo MJ, Sousa JM, Pozo-Borrego AJ, Núñez-Roldán A. IgG subclass profile among anti-Glutathione S-transferase T1 antibodies in post-transplant de novo immune hepatitis. *Clin Transplant* 2016; **30**: 210-217 [PMID: 26663521 DOI: 10.1111/ctr.12675]

62 **Ekong UD**, Mathew J, Melin-Aldana H, Wang D, Alonso EM. Successful resolution of inflammation and increased regulatory T cells in sirolimus-treated post-transplant allograft hepatitis. *Pediatr Transplant* 2012; **16**: 165-175 [PMID: 22360400 DOI: 10.1111/j.1399-3046.2012.01648.x]

63 **Edmunds C**, Ekong UD. Autoimmune Liver Disease Post-Liver Transplantation: A Summary and Proposed Areas for Future Research. *Transplantation* 2016; **100**: 515-524 [PMID: 26447505 DOI: 10.1097/TP.0000000000000922]

64 **Martínez-Bravo MJ**, Calderón-Cabrera C, Márquez-Malaver FJ, Rodríguez N, Guijarro M, Espigado I, Núñez-Roldán A, Pérez-Simón JA, Aguilera I. Mismatch on glutathione S-transferase T1 increases the risk of graft-versus-host disease and mortality after allogeneic stem cell transplantation. *Biol Blood Marrow Transplant* 2014; **20**: 1356-1362 [PMID: 24844856 DOI: 10.1016/j.bbmt.2014.05.008]

65 **Aguado-Dominguez E**, Sousa JM, Perez-Simon JA, Aguilera I. Clinical association of anti-glutathione S-transferase T1 antibodies and de novo immune hepatitis after hematopoietic cell transplantation. *Dig Liver Dis* 2018; **50**: 418-419 [PMID: 29409781 DOI: 10.1016/j.dld.2017.12.033]

66 **Kerkar N**, Yanni G. 'De novo' and 'recurrent' autoimmune hepatitis after liver transplantation: A comprehensive review. *J Autoimmun* 2016; **66**: 17-24 [PMID: 26377632 DOI: 10.1016/j.jaut.2015.08.017]

67 **Arterbery AS**, Osafo-Addo A, Avitzur Y, Ciarleglio M, Deng Y, Lobritto SJ, Martinez M, Hafler DA, Kleinewietfeld M, Ekong UD. Production of Proinflammatory Cytokines by Monocytes in Liver-Transplanted Recipients with De Novo Autoimmune Hepatitis Is Enhanced and Induces TH1-like Regulatory T Cells. *J Immunol* 2016; **196**: 4040-4051 [PMID: 27183637 DOI: 10.4049/jimmunol.150227]

68 **Aguilera I**, Sousa JM, Praena JM, Gómez-Bravo MA, Núñez-Roldan A. Choice of calcineurin inhibitor may influence the development of de novo immune hepatitis associated with anti-GSTT1 antibodies after liver transplantation. *Clin Transplant* 2011; **25**: 207-212 [PMID: 20236132 DOI: 10.1111/j.1399-0012.2010.01221.x]

Endoscopic diagnosis of sessile serrated adenoma/polyp with and without dysplasia/carcinoma

Takashi Murakami, Naoto Sakamoto, Akihito Nagahara

Takashi Murakami, Naoto Sakamoto, Akihito Nagahara, Department of Gastroenterology, Juntendo University School of Medicine, Tokyo 113-8421, Japan

ORCID number: Takashi Murakami (0000-0001-7419-589X); Naoto Sakamoto (0000-0002-2143-0734); Akihito Nagahara (0000-0001-5979-4384).

Author contributions: Murakami T mainly contributed to this work, generated the figures and wrote the manuscript; Sakamoto N and Nagahara A contributed equally to the writing of the manuscript.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Abstract

Sessile serrated adenoma/polyps (SSA/Ps) are early precursor lesions in the serrated neoplasia pathway, which results in colorectal carcinomas with *BRAF* mutations, methylation for DNA repair genes, a CpG island methylator phenotype, and high levels of microsatellite instability. Some of these lesions can rapidly become dysplastic or invasive carcinomas that exhibit high lymphatic invasion and lymph node metastasis potentials. Detecting serrated lesions, including SSA/Ps with and without dysplasia/carcinoma, is critical, but SSA/Ps can be difficult to detect, are inconsistently identified by endoscopists and pathologists, and are often incompletely resected. Therefore, SSA/Ps are considered to be major contributors to "interval cancers". If colonoscopists can identify the specific endoscopic characteristics of SSA/Ps, their detection and the effectiveness of colonoscopy may improve. Here, the endoscopic features of SSA/Ps with and without dysplasia/carcinoma, including the characteristics determined using magnifying endoscopy, are reviewed in the context of previous reports. Endoscopically, these subtle polyps are like hyperplastic polyps, because they are slightly elevated and pale. Unlike hyperplastic polyps, SSA/Ps are usually larger than 5 mm, frequently covered by a thin layer called the "mucus cap", and are more commonly located in the proximal colon. Magnifying narrow-band imaging findings, which include dark spots inside the crypts and varicose microvascular vessels, in addition to the type II-open pit patterns detected using magnifying chromoendoscopy, effectively differentiate SSA/Ps from hyperplastic polyps. The lesions' endoscopic characteristics, which include their (semi)pedunculated morphologies, double elevations, central depressions, and reddishness, and the use of magnifying endoscopy, might help to detect dysplasia/carcinoma within SSA/Ps. Greater awareness may promote further research into improving the detection, identification, and complete

resection rates of SSA/Ps with and without dysplasia/carcinoma and reduce the interval cancer rates.

Core tip: The endoscopic features of sessile serrated adenoma/polyps (SSA/Ps) with and without dysplasia/carcinoma are reviewed. Conventional endoscopic characteristics, including a proximal location, a slightly elevated morphology, a pale color, and a mucus cap, are useful for diagnosing SSA/Ps. Magnifying narrow-band imaging, which detects dark spots inside the crypts and varicose microvascular vessels, and magnifying chromoendoscopy, which identifies the type II-open pit pattern, are also effective for differentiating between SSA/Ps and hyperplastic polyps. Furthermore, the lesions' endoscopic characteristics, which include their (semi)pedunculated morphologies, double elevations, central depressions, and reddishness, and the use of magnifying endoscopy, might help to detect dysplasia/carcinoma within SSA/Ps.

INTRODUCTION

Colorectal serrated lesions were called "hyperplastic polyps", and they were not considered to be malignant[1,2]. Torlakovic *et al*[3] described abnormal proliferations in colorectal serrated polyps that resembled hyperplastic polyps superficially, but could be distinguished histologically based on their abnormal architectural features, and they introduced the term "sessile serrated adenoma". Currently, these polyps are categorized as sessile serrated adenoma/polyp (SSA/P) in accordance with the World Health Organization's recommendations[4]. The typical histology of an SSA/P in a representative case is shown in Figure 1.

SSA/Ps are early precursor lesions in the serrated neoplasia pathway, which results in colorectal carcinomas with high levels of microsatellite instability[5-7]. Recent studies have shown associations between SSA/Ps with and without dysplasia or carcinoma and the methylation or loss of protein expression for DNA repair genes, including *MLH1*[3,6,8-12], a CpG island methylator phenotype[5,6,8,10], *BRAF* mutations[5,6,8-17], and a lack of genetic alterations in *CTNNB1*, which is the gene that
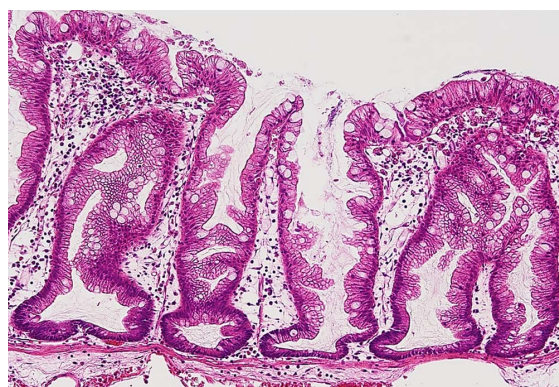

Figure 1 Typical histology of a sessile serrated adenoma/polyp. Crypts with a serrated architecture include those that are irregularly dilated, branch irregularly, and are horizontally arranged (basal).

codes for β-catenin protein[17]. This pathway is thought to be distinct from the conventional adenoma-carcinoma pathway in which adenomas progress to invasive colorectal carcinomas as a result of a series of genetic alterations, including adenomatous polyposis coli (*APC*) and *KRAS* mutations[6,8,13,14,18,19].

Some researchers[20-22] have suggested that some serrated lesions might progress rapidly to dysplasia or invasive carcinomas. Furthermore, we reported that the submucosal invasive carcinomas that arose in SSA/Ps exhibited higher potentials for lymphatic invasion and lymph node metastasis than their conventional counterparts that arose from tubular adenomas[23]. Therefore, the detection of serrated lesions, including SSA/Ps with and without dysplasia, is critical. However, SSA/Ps can be difficult to detect, are inconsistently identified by endoscopists and pathologists, and are often incompletely resected[24-27]. Therefore, SSA/Ps are major contributors to the failure of colonoscopy to prevent proximal colonic cancer[28-30], and they account for 5%-7% of the colorectal cancers that occur in the interval between a complete colonoscopy and surveillance, that is, "interval cancer"[31-33]. The identification of the specific endoscopic characteristics of SSA/Ps by colonoscopists may improve their detection and, eventually, may enhance the effectiveness of colonoscopy. Some studies have investigated the endoscopic features of SSA/Ps without dysplasia[34-38], and we clarified the endoscopic characteristics of SSA/Ps that had advanced histology[39].

Here, the endoscopic features of SSA/Ps with and without dysplasia or carcinoma are reviewed in the context of previous reports, including the features detected using magnifying endoscopy.

DIAGNOSIS OF SSA/P USING CONVENTIONAL WHITE-LIGHT ENDOSCOPY

Generally, hyperplastic polyps are traditionally considered non-neoplastic, but SSA/Ps have malignant potential to progress to invasive carcinomas. Therefore,
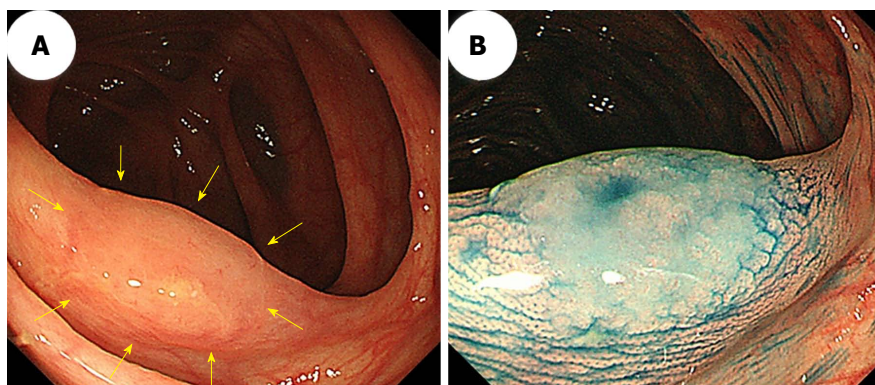
Figure 2 A sessile serrated adenoma/polyp in the transverse colon that measured 13 mm. A: An image from conventional colonoscopy showing the lesion's location (arrows); B: An image from chromoendoscopy following indigo carmine dye spraying.

differentiating an SSA/P from a hyperplastic polyp is clinically important to determine the necessity of an endoscopic resection or to provide support for a recommendation of a surveillance interval[40,41]. Typical hyperplastic polyps are highly prevalent, diminutive sessile polyps that are most commonly located in the sigmoid colon and rectum, and identifying them endoscopically is not particularly difficult[42]. SSA/Ps are subtle polyps, and their endoscopic findings are similar to those associated with hyperplastic polyps, which include a slightly elevated morphology and a pale color. However, in contrast to hyperplastic polyps, SSA/Ps are usually larger than 5 mm, frequently covered by a thin layer called a "mucus cap"[4,34,43,44], and are more commonly located in the proximal colon[14,45]. Conversely, although SSA/Ps are difficult to detect because of their slightly elevated morphology, adhesion of mucus in the proximal colon can be one of the most useful clues for SSA/P detection. Additionally, using white-light endoscopy, Hazewinkel *et al*[37] described the presence of indistinct borders and a cloud-like surface, and showed that these were independently predictive endoscopic characteristics that were associated with the histology of SSA/Ps. Figure 2 shows representative endoscopic images of SSA/Ps.

ENDOSCOPIC DIAGNOSIS OF SSA/P USING NARROW-BAND IMAGING

Difficulties distinguishing between an SSA/P and a hyperplastic polyp are commonly encountered. Many authors have used image-enhanced endoscopy to characterize polyps[46], which involves the use of innovative optical technologies, such as narrow-band imaging (NBI)[47-50]. Bile appears as a bright red fluid using NBI. When a tenacious mucus cap covers SSA/Ps, the mucus cap is clearly viewed using NBI. Therefore, NBI enhances the visibility of SSA/Ps that have mucus caps, which are usually an intense red color[34] (Figure 3A and B).

Furthermore, NBI often reveals small dark dots inside the openings to the crypts of SSA/Ps[37]; these are thought to indicate crypt dilations, which are a key

histological feature of SSA/Ps. The presence of these dark spots inside the crypts might help endoscopists to differentiate between premalignant SSA/Ps and hyperplastic polyps during colonoscopy[37,38] (Figure 3C and D). Hazewinkel *et al*[37] have reported that in white-light endoscopy, indistinctive borders and cloud-like surface are two independent predictive characteristics of SSA/P, while in NBI, it is possible to discern an irregular shape and dark spots inside the crypts. The sensitivities, specificities, and overall accuracies determined using white-light endoscopy were 75%, 79%, and 77%, respectively, and those determined using NBI were 89%, 96%, and 93%, respectively[37].

Magnifying NBI can enhance the visibility of the microvessels on a lesion's surface. Yamada *et al*[51] conducted a multivariate analysis, demonstrated that dilated and branching vessels, defined as thickened capillary vessels with branching that is observed on the surface, had a 2.3-fold odds ratio among SSA/Ps compared with hyperplastic polyps. They stated that when dilated and branching vessels, a proximal location, and a tumor size of ≥ 10 mm were combined, the positive predictive value exceeded 90%. Additionally, Uraoka *et al*[52] reported that the presence of varicose microvascular vessels, which were found using magnifying NBI, was useful for differentiating between SSA/Ps and hyperplastic polyps. Unlike the blood vessels around the glands of the superficial mucosal layer such as dilated and branching vessels, varicose microvascular vessels are characterized by the observation of blood vessels running throughout the deep mucosal layer. The presence of varicose microvascular vessels had a significantly higher specificity (88%) for predicting a diagnosis of SSA/P (Figure 3E and F).

DIAGNOSIS OF SSA/P USING MAGNIFYING CHROMOENDOSCOPY

Magnifying chromoendoscopy, which uses indigo carmine or crystal violet staining, follows careful conventional endoscopic examinations. Kudo *et al*[53,54] proposed a classification of colorectal lesions' pit patterns that is associated with the lesions' histologic characteristics.

Table 1 Distinct endoscopic characteristics between sessile serrated adenoma/polyps and hyperplastic polyps

	SSA/Ps	Hyperplastic polyps
Conventional endoscopic features		
Location	Proximal	Distal
Size of tumor	> 5 mm	≤ 5 mm
Color	Pale	Pale
Morphology	Flat elevated	Flat elevated
Mucus cap	Yes	No
Endoscopic features by using NBI	Irregular shape	-
	Small dark dots	
	Dilated and branching vessels	
	Varicose microvascular vessels	
Magnifying chromoendoscopic features	Type II-open pit pattern	Type II pit pattern

SSA/P: Sessile serrated adenoma/polyp; NBI: Narrow-band imaging.

As previously explained[54-56], magnifying colonoscopy is useful for differentiating between neoplastic and nonneoplastic lesions, and for assessing early colorectal cancers' depths of invasion. Both hyperplastic polyps and SSA/Ps have type II pit patterns. Recently, the type II-open pit pattern has been described as a hallmark of SSA/Ps (sensitivity: 66%; specificity: 97%)[35]. Like the small dark dots detected using NBI, a type II-open pit pattern detected using magnifying chromoendoscopy is thought to indicate crypt dilation, which is one of the major histological features of SSA/Ps (Figure 4).

Distinct endoscopic characteristics between SSA/Ps and hyperplastic polyps are summarized in Table 1.

ENDOSCOPIC DETECTION OF SSA/P

The detection of SSA/Ps requires careful colonoscopy. As stated above, because most SSA/Ps are slightly flat-elevated and have subtle mucosal features, SSA/Ps are difficult to detect with endoscopy, and could easily be missed. Therefore, bowel preparation must be excellent. Potential SSA/Ps are initially considered at long view and investigated at close-up view. At long view, the presence of SSA/P is suspected when there is a patch that appears nodular, reddish, covered with mucus, and/or circled by fine debris. Then such a lesion must be approached and the mucosa washed. Finally, at close-up view, using white light and under NBI, the surface pattern and vessels are examined.

Recently, some studies[57,58] have shown that image-enhanced endoscopy such as NBI might increase the detection of serrated lesions in the proximal colon, although the results did not reach significance. Therefore, image-enhanced endoscopy currently cannot be recommended as a detection tool for SSA/P. Additional studies assessing SSA/P detection rates with image-enhanced endoscopy are needed.

ENDOSCOPIC DIAGNOSIS OF SSA/P WITH DYSPLASIA/CARCINOMA

SSA/Ps with advanced histology, including cytologic dysplasia or minimally invasive carcinomas, are rare.

Indeed, a previous study's findings showed that the frequencies of cytologic dysplasia and invasive carcinomas among SSA/P lesions were 14% and 1.0%, respectively[59]. The findings from another study showed that three (0.7%) high-grade dysplasias and one (0.2%) submucosal invasive carcinoma were detected among 430 SSA/Ps[60]. Therefore, only a few studies have investigated the endoscopic characteristics of SSA/Ps with dysplasia or carcinoma in detail[39,61,62]. We demonstrated that SSA/Ps without dysplasia (354 of 414; 86%) and SSA/Ps with dysplasia or carcinomas (40 of 48; 83%) were frequently located in the proximal colon[39]. Furthermore, we showed a stepwise increase in the median size of the SSA/Ps that accompanied their dysplastic progression, specifically, from a 10-mm SSA/P that did not have dysplasia to a 12-mm SSA/P with cytologic dysplasia and a 19-mm SSA/P with an invasive carcinoma, but 19 of 48 (39.6%) SSA/Ps with dysplasia or carcinomas measured ≤ 10 mm[39]. The findings from a study by Goldstein[20] showed that the median size of eight SSA/Ps with focal invasive adenocarcinomas or high-grade dysplasia was 8.5 mm (range: 6-12 mm). Another study's findings[63] showed that among eight SSA/Ps with intramucosal carcinomas, submucosal carcinomas, or advanced carcinomas, the largest diameter was ≤ 10 mm. Therefore, SSA/P with dysplasia/carcinoma must be attended to even if the lesion measures 10 mm or less.

Macroscopically, a mucus cap was found in almost all of the SSA/P lesions, including the SSA/Ps with and without dysplasia or carcinoma, in our study[39], suggesting that a mucus cap may be one of the strongest markers of an SSA/P. Additionally, (semi)pedunculated morphologies, double elevations, central depressions, and reddishness were found more frequently in SSA/Ps with dysplasia (17.1%, 63.4%, 9.8%, and 39.0%, respectively) or carcinoma (28.6%, 57.1%, 28.6%, and reddishness 85.7%, respectively) than the frequencies at which these features were found in SSA/Ps without dysplasia (4.6%, 4.6%, 3.9%, and 3.4%, respectively). The presence of at least one of these four markers had a high sensitivity (91.7%) for the identification of dysplasia or a carcinoma within an SSA/P; the specificity was
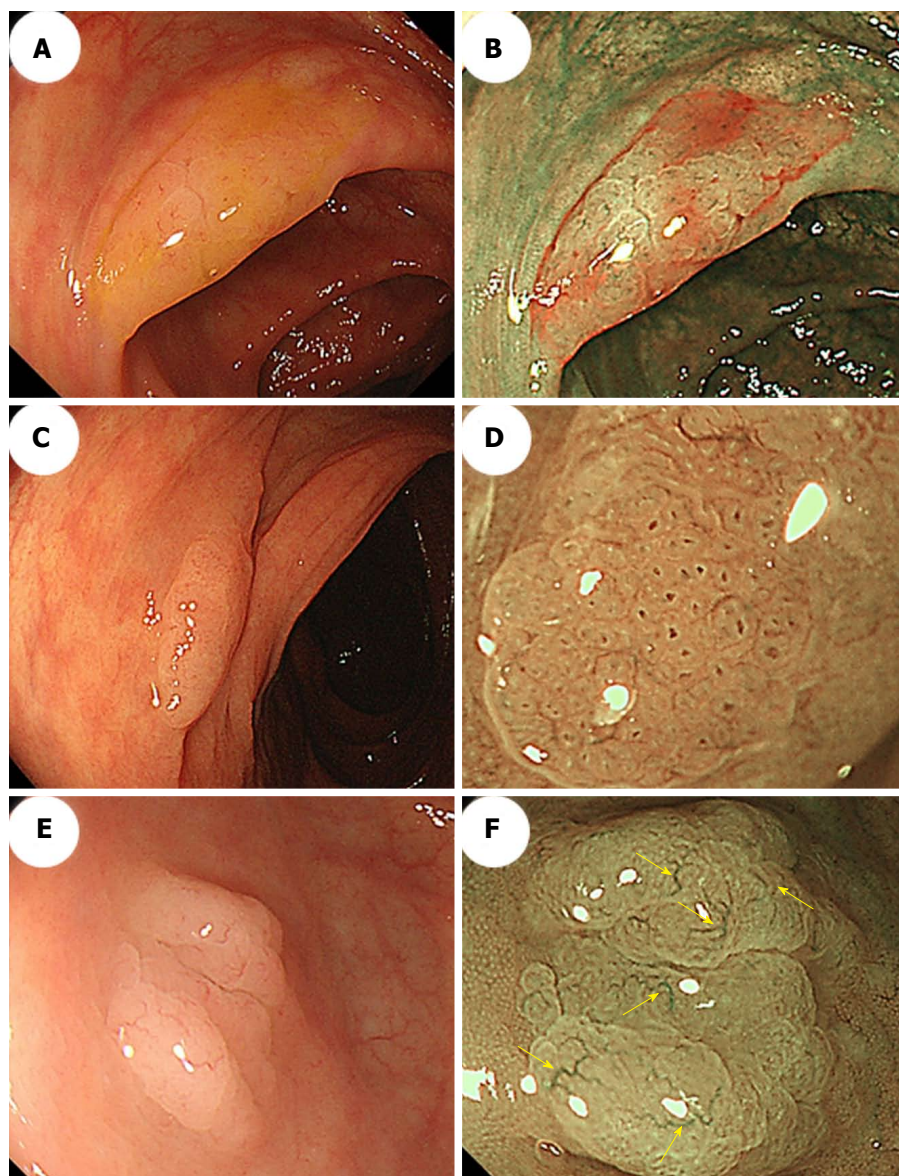
Figure 3 Morphologic characteristics of sessile serrated adenoma/polyps. A: Conventional endoscopy revealed a flat-elevated lesion with a 20-mm diameter that was covered with a mucus cap in the transverse colon. B: Narrow-band imaging (NBI) showed that the SSA/P in (A) was covered with a mucus cap that appeared intensely red. C: Conventional endoscopy showed a flat-elevated lesion with a 14-mm diameter in the ascending colon. D: Magnifying NBI of the SSA/P in (C) revealed dark spots inside the crypts in part of the lesion. E: A conventional endoscopic image shows a flat-elevated pale colored lesion with a 10-mm diameter in the cecum. F: Magnifying NBI of the SSA/P in (E) revealed varicose microvascular vessels (arrows) in part of the lesion. SSA/P: Sessile serrated adenoma/polyp.
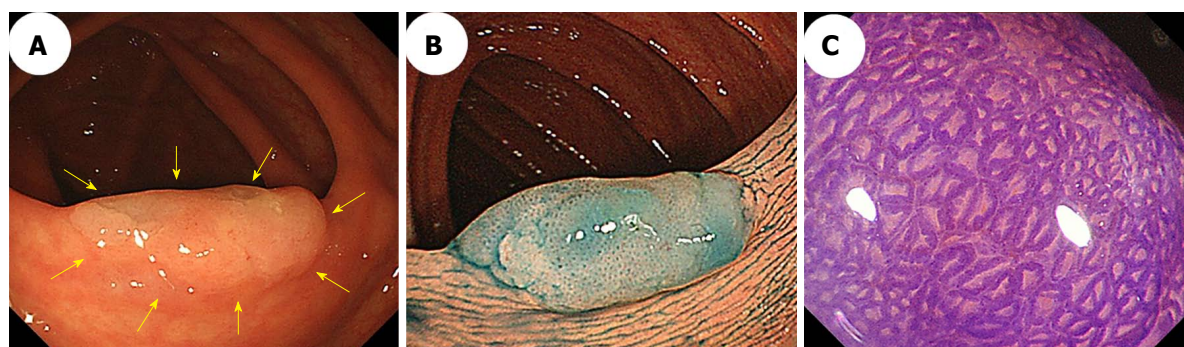

Figure 4 Conventional colonoscopic image (A) and a chromoendoscopic image (B) following indigo carmine dye spraying show an 18-mm sessile serrated adenoma/polyp with a mucus cap that was in the transverse colon (arrows). C: Magnifying chromoendoscopy using crystal violet staining identified a type II-open pit pattern in the lesion.
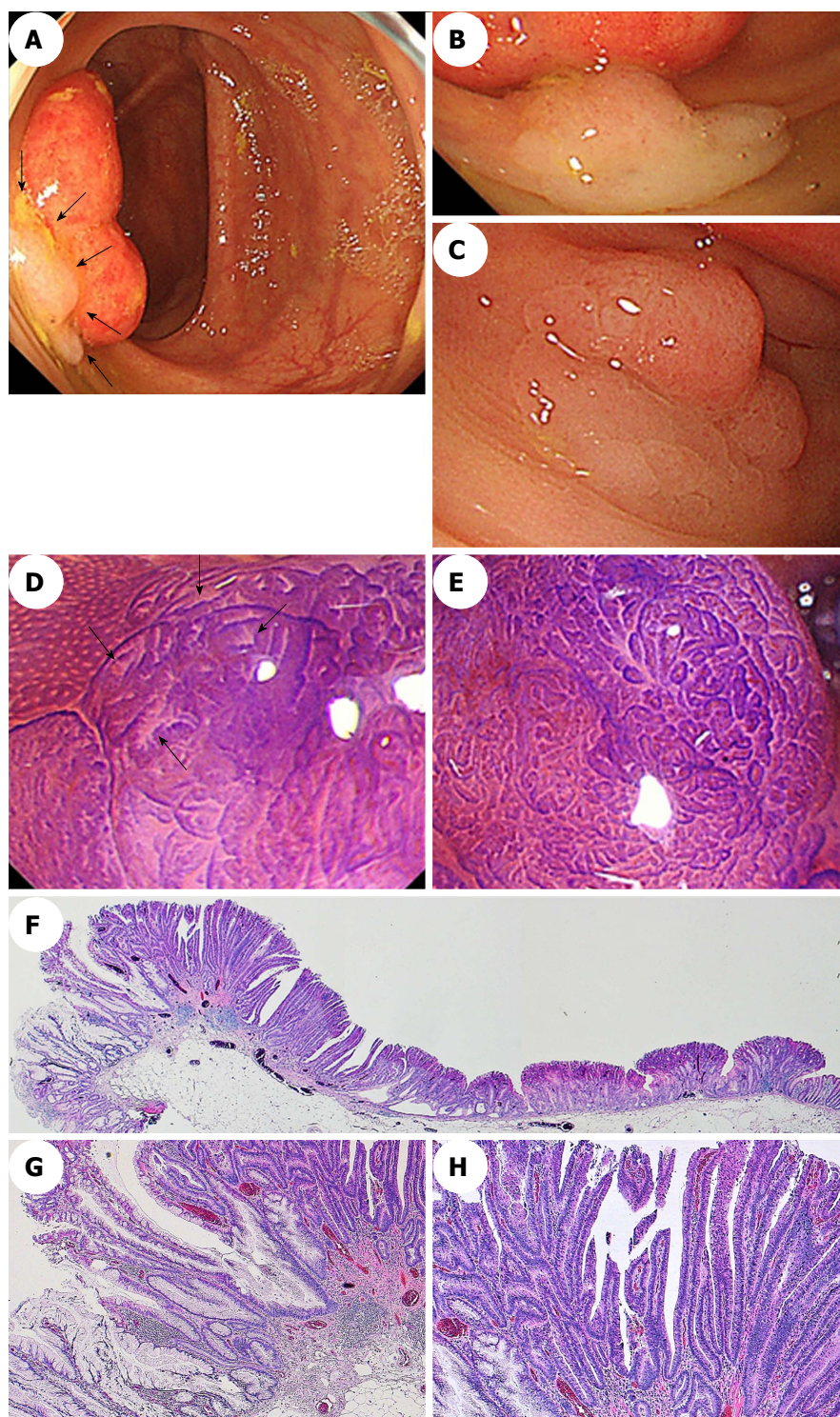
Figure 5 Endoscopic images of a sessile serrated adenoma/polyp with high-grade cytologic dysplasia in a representative case. A-C: A conventional endoscopic view using white-light imaging. A: An endoscopic image shows a pale-color, flat-elevated lesion covered with mucus at the ascending colon (arrows). B: The lesion is covered with mucus cap. C: After washing the target lesion to sufficiently remove mucus, a flat-elevated lesion that had a 13-mm diameter and a dome-shaped double elevation can be clearly seen. The dome-shaped area is slightly red-colored. D and E: Magnifying chromoendoscopic views using crystal violet staining. D: A type II-open pit pattern is partly evident in the edge of the lesion (arrows). E: Type V_I-mild pit pattern consisting of areas with irregular pits can be observed at the dome-shaped area. We endoscopically diagnosed the lesion as an SSA/P with cytologic dysplasia, and achieved an en bloc resection by performing an endoscopic mucosal resection. F-H: Histopathologic findings with hematoxylin-eosin staining of the resected specimen. G: Crypts with a serrated architecture exhibit irregularly dilated crypts, irregularly branching crypts, and horizontally arranged basal crypts, corresponding to SSA/P. H: A high-power view shows conventional adenomatous high-grade dysplasia with cytological atypia and architectural dysplasia in the dome-shaped area. The lesion was pathologically consistent with an SSA/P with high-grade cytologic dysplasia. SSA/P: Sessile serrated adenoma/polyp.
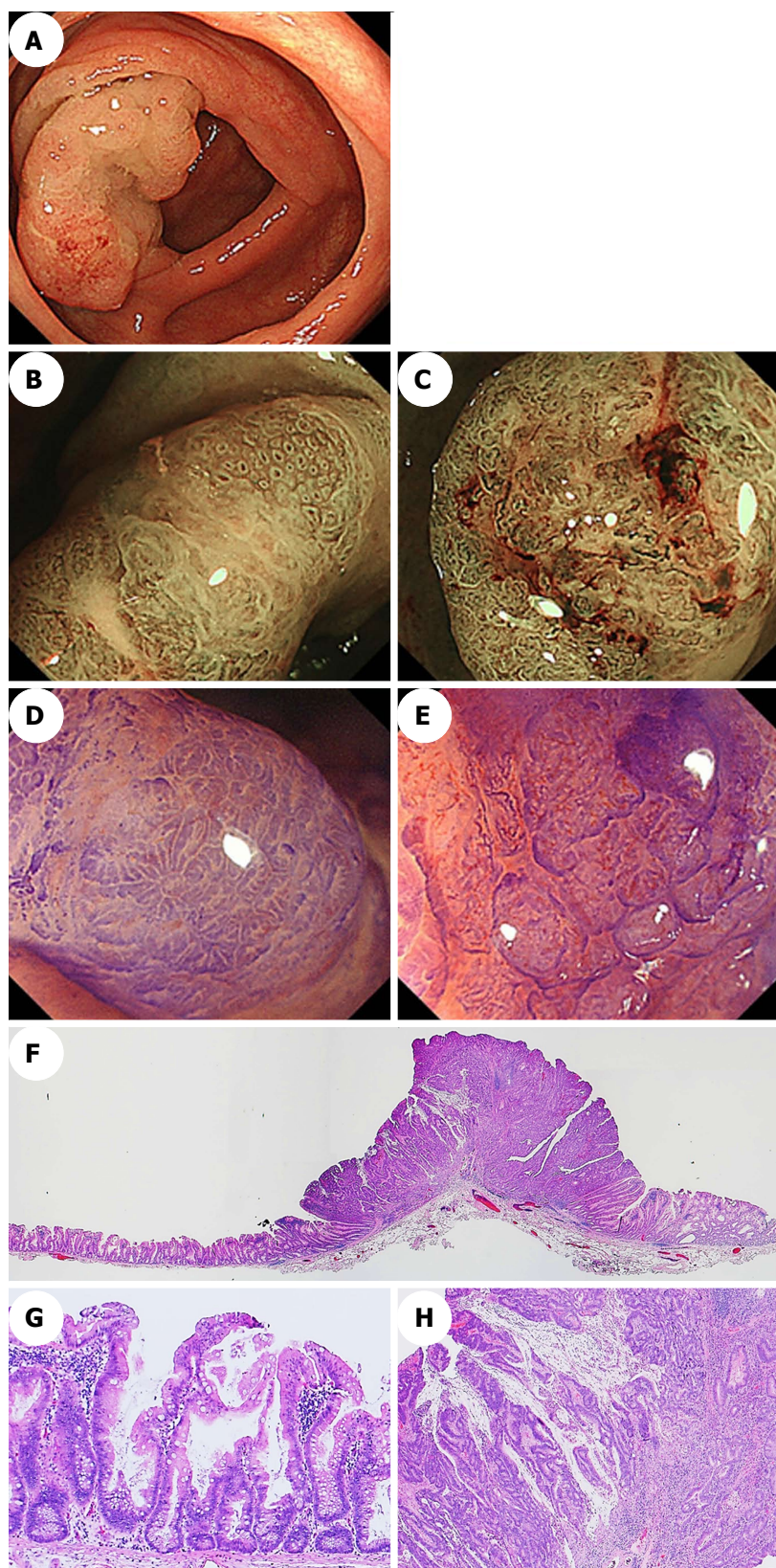
Figure 6 Endoscopic images of a sessile serrated adenoma/polyp with an invasive carcinoma in a representative case. A: A conventional endoscopic image captured using white-light imaging shows a red 55-mm semipedunculated lesion in the ascending colon. B and C: Magnifying narrow-band imaging revealed dark spots inside the crypts on an edge of the lesion and irregular vessel patterns over a large part of the lesion, respectively. D and E: Magnifying chromoendoscopy using crystal violet staining; D: A high-powered view of the marginal zone, the dilated openings of the crypts have a type II-open pit pattern; E: A high-powered view of the middle region in which a type V$_I$-severe pit pattern is evident. We endoscopically diagnosed the lesion as a carcinoma associated with an SSA/P, and achieved an en bloc resection by performing an endoscopic submucosal dissection. F-H: Histopathologic findings with hematoxylin-eosin staining of the resected specimen; G: Crypts with a serrated architecture exhibiting irregularly dilated crypts and irregularly branching crypts, corresponding to SSA/P; H: Well to moderately differentiated adenocarcinomas invade the submucosa with extracellular mucin production. The lesion was pathologically consistent with an invasive submucosal adenocarcinoma associated with an SSA/P. SSA/P: Sessile serrated adenoma/polyp.

85.3%. These findings suggested that the endoscopic characteristics, including a (semi)pedunculated morphology, a double elevation, a central depression, and reddishness, may be useful for accurately diagnosing the presence of advanced histology within an SSA/P.

Magnifying chromoendoscopy is also useful for detecting components associated with dysplasia or a carcinoma within an SSA/P. We found that a type II-open pit pattern was present in SSA/Ps without dysplasia and in SSA/Ps with dysplasia or carcinomas, which indicates that a type II-open pit pattern may be strongly suggestive of the presence of SSA/P components[39]. Furthermore, the type II pit pattern only was detected in all of the cases who had SSA/Ps without dysplasia, whereas type II and other pit patterns, including mixtures of III$_L$, IV, V$_I$, or V$_N$, were found in most of the SSA/Ps with dysplasia or carcinoma. Moreover, all of the cases who had SSA/Ps with invasive carcinomas had the V$_I$ or V$_N$ pit patterns (invasive patterns), which were consistent with the depths of invasion. Accordingly, determining the pit patterns using magnifying endoscopy can effectively assess the depth of invasion of early colorectal cancers that arise from SSA/Ps. Figures 5 and 6 show representative endoscopic images of SSA/Ps with dysplasia or carcinoma.

Finally, there is one important point that must be kept in mind when observing SSA/Ps using colonoscopy. Most SSA/Ps were covered with rich mucus, and subtle endoscopic findings were difficult to detect when sticky mucus was present. After washing the target lesion to sufficiently remove mucus, endoscopic findings such as (semi)pedunculated morphology, double elevation, central depression, and reddishness should be assessed, and pit pattern analysis must be performed.

CONCLUSION

Conventional endoscopic characteristics, including a proximal location, a slightly elevated morphology, a pale color, and a mucus cap, are useful for diagnosing SSA/Ps. Magnifying endoscopy with NBI, which detects dark spots inside the crypts and varicose microvascular vessels, and magnifying chromoendoscopy, which identifies the type II-open pit pattern, are also effective for differentiating between SSA/Ps and hyperplastic polyps. Furthermore, a lesion's endoscopic characteristics, for example, a (semi)pedunculated morphology, a double elevation, a central depression, and reddishness, in addition to the use of magnifying endoscopy, might be useful for identifying dysplasia or a carcinoma within an SSA/P. Greater awareness may promote further research into improving the detection, recognition, and complete resection rates of SSA/Ps with and without dysplasia or carcinoma and reduce the interval cancer rates.

REFERENCES

1   **Lane N**. The precursor tissue of ordinary large bowel cancer. *Cancer Res* 1976; **36**: 2669-2672 [PMID: 1277173]

2   **Jørgensen H**, Mogensen AM, Svendsen LB. Hyperplastic polyposis of the large bowel. Three cases and a review of the literature. *Scand J Gastroenterol* 1996; **31**: 825-830 [PMID: 8858755]

3   **Torlakovic E**, Skovlund E, Snover DC, Torlakovic G, Nesland JM. Morphologic reappraisal of serrated colorectal polyps. *Am J Surg Pathol* 2003; **27**: 65-81 [PMID: 12502929]

4   **Snover DC**, Ahnen DJ, Burt RW. Serrated polyps of the colon and rectum and serrated polyposis. In: Bosman FT, Carneiro F, Hruban RH, Theise ND, eds. WHO classification of tumours of the digestive system. Lyon: IARC Press; 2010: 160-165

5   **Kambara T**, Simms LA, Whitehall VL, Spring KJ, Wynter CV, Walsh MD, Barker MA, Arnold S, McGivern A, Matsubara N, Tanaka N, Higuchi T, Young J, Jass JR, Leggett BA. BRAF mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. *Gut* 2004; **53**: 1137-1144 [PMID: 15247181 DOI: 10.1136/gut.2003.037671]

6   **O'Brien MJ**, Yang S, Mack C, Xu H, Huang CS, Mulcahy E, Amorosino M, Farraye FA. Comparison of microsatellite instability, CpG island methylation phenotype, BRAF and KRAS status in serrated polyps and traditional adenomas indicates separate pathways to distinct colorectal carcinoma end points. *Am J Surg Pathol* 2006; **30**: 1491-1501 [PMID: 17122504 DOI: 10.1097/01.pas.0000213313.36306.85]

7   **Patil DT**, Shadrach BL, Rybicki LA, Leach BH, Pai RK. Proximal colon cancers and the serrated pathway: a systematic analysis of precursor histology and BRAF mutation status. *Mod Pathol* 2012; **25**: 1423-1431 [PMID: 22684223 DOI: 10.1038/modpathol.2012.98]

8   **Kim YH**, Kakar S, Cun L, Deng G, Kim YS. Distinct CpG island methylation profiles and BRAF mutation status in serrated and adenomatous colorectal polyps. *Int J Cancer* 2008; **123**: 2587-2593 [PMID: 18798261 DOI: 10.1002/ijc.23840]

9   **Sandmeier D**, Benhattar J, Martin P, Bouzourene H. Serrated polyps of the large intestine: a molecular study comparing sessile serrated adenomas and hyperplastic polyps. *Histopathology* 2009; **55**: 206-213 [PMID: 19694828 DOI: 10.1111/j.1365-2559.2009.03356.x]

10 **Kim KM**, Lee EJ, Ha S, Kang SY, Jang KT, Park CK, Kim JY, Kim YH, Chang DK, Odze RD. Molecular features of colorectal hyperplastic polyps and sessile serrated adenoma/polyps from Korea. *Am J Surg Pathol* 2011; **35**: 1274-1286 [PMID: 21836485 DOI: 10.1097/PAS.0b013e318224cd2e]

11 **Dhir M**, Yachida S, Van Neste L, Glöckner SC, Jeschke J, Pappou EP, Montgomery EA, Herman JG, Baylin SB, Iacobuzio-Donahue C, Ahuja N. Sessile serrated adenomas and classical adenomas: an epigenetic perspective on premalignant neoplastic lesions of the gastrointestinal tract. *Int J Cancer* 2011; **129**: 1889-1898 [PMID: 21154739 DOI: 10.1002/ijc.25847]

12 **Murakami T**, Mitomi H, Saito T, Takahashi M, Sakamoto N, Fukui N, Yao T, Watanabe S. Distinct WNT/β-catenin signaling activation in the serrated neoplasia pathway and the adenoma-carcinoma sequence of the colorectum. *Mod Pathol* 2015; **28**: 146-158 [PMID: 24925057 DOI: 10.1038/modpathol.2014.41]

13 **Jass JR**, Baker K, Zlobec I, Higuchi T, Barker M, Buchanan D, Young J. Advanced colorectal polyps with the molecular and morphological features of serrated polyps and adenomas: concept of a 'fusion' pathway to colorectal cancer. *Histopathology* 2006; **49**: 121-131 [PMID: 16879389 DOI: 10.1111/j.1365-2559.2006.02466.x]

14 **Spring KJ**, Zhao ZZ, Karamatic R, Walsh MD, Whitehall VL, Pike T, Simms LA, Young J, James M, Montgomery GW, Appleyard M, Hewett D, Togashi K, Jass JR, Leggett BA. High prevalence of sessile serrated adenomas with BRAF mutations: a prospective study of patients undergoing colonoscopy. *Gastroenterology* 2006; **131**: 1400-1407 [PMID: 17101316 DOI: 10.1053/j.gastro.2006.08.038]

15 **Carr NJ**, Mahajan H, Tan KL, Hawkins NJ, Ward RL. Serrated and non-serrated polyps of the colorectum: their prevalence in an unselected case series and correlation of BRAF mutation analysis with the diagnosis of sessile serrated adenoma. *J Clin Pathol* 2009; **62**: 516-518 [PMID: 19126563 DOI: 10.1136/jcp.2008.061960]

16 **Fujita K**, Yamamoto H, Matsumoto T, Hirahashi M, Gushima

M, Kishimoto J, Nishiyama K, Taguchi T, Yao T, Oda Y. Sessile serrated adenoma with early neoplastic progression: a clinicopathologic and molecular study. *Am J Surg Pathol* 2011; **35**: 295-304 [PMID: 21263251 DOI: 10.1097/PAS.0b013e318205df36]

17 **Yachida S**, Mudali S, Martin SA, Montgomery EA, Iacobuzio-Donahue CA. Beta-catenin nuclear labeling is a common feature of sessile serrated adenomas and correlates with early neoplastic progression after BRAF activation. *Am J Surg Pathol* 2009; **33**: 1823-1832 [PMID: 19745699 DOI: 10.1097/PAS.0b013e3181b6da19]

18 **Powell SM**, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, Vogelstein B, Kinzler KW. APC mutations occur early during colorectal tumorigenesis. *Nature* 1992; **359**: 235-237 [PMID: 1528264 DOI: 10.1038/359235a0]

19 **Miyoshi Y**, Nagase H, Ando H, Horii A, Ichii S, Nakatsuru S, Aoki T, Miki Y, Mori T, Nakamura Y. Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. *Hum Mol Genet* 1992; **1**: 229-233 [PMID: 1338904]

20 **Goldstein NS**. Small colonic microsatellite unstable adenocarcinomas and high-grade epithelial dysplasias in sessile serrated adenoma polypectomy specimens: a study of eight cases. *Am J Clin Pathol* 2006; **125**: 132-145 [PMID: 16483002]

21 **Snover DC**. Update on the serrated pathway to colorectal carcinoma. *Hum Pathol* 2011; **42**: 1-10 [PMID: 20869746 DOI: 10.1016/j.humpath.2010.06.002].]

22 **Bettington M**, Walker N, Clouston A, Brown I, Leggett B, Whitehall V. The serrated pathway to colorectal carcinoma: current concepts and challenges. *Histopathology* 2013; **62**: 367-386 [PMID: 23339363 DOI: 10.1111/his.12055]

23 **Murakami T**, Mitomi H, Yao T, Saito T, Shibuya T, Sakamoto N, Osada T, Watanabe S. Distinct histopathological characteristics in colorectal submucosal invasive carcinoma arising in sessile serrated adenoma/polyp and conventional tubular adenoma. *Virchows Arch* 2018; **472**: 383-393 [PMID: 28929387 DOI: 10.1007/s00428-017-2234-8]

24 **Hetzel JT**, Huang CS, Coukos JA, Omstead K, Cerda SR, Yang S, O'Brien MJ, Farraye FA. Variation in the detection of serrated polyps in an average risk colorectal cancer screening cohort. *Am J Gastroenterol* 2010; **105**: 2656-2664 [PMID: 20717107 DOI: 10.1038/ajg.2010.315]

25 **Kahi CJ**, Hewett DG, Norton DL, Eckert GJ, Rex DK. Prevalence and variable detection of proximal colon serrated polyps during screening colonoscopy. *Clin Gastroenterol Hepatol* 2011; **9**: 42-46 [PMID: 20888435 DOI: 10.1016/j.cgh.2010.09.013]

26 **Pohl H**, Srivastava A, Bensen SP, Anderson P, Rothstein RI, Gordon SR, Levy LC, Toor A, Mackenzie TA, Rosch T, Robertson DJ. Incomplete polyp resection during colonoscopy-results of the complete adenoma resection (CARE) study. *Gastroenterology* 2013; **144**: 74-80.e1 [PMID: 23022496 DOI: 10.1053/j.gastro.2012.09.043]

27 **de Wijkerslooth TR**, Stoop EM, Bossuyt PM, Tytgat KM, Dees J, Mathus-Vliegen EM, Kuipers EJ, Fockens P, van Leerdam ME, Dekker E. Differences in proximal serrated polyp detection among endoscopists are associated with variability in withdrawal time. *Gastrointest Endosc* 2013; **77**: 617-623 [PMID: 23321338 DOI: 10.1016/j.gie.2012.10.018]

28 **Baxter NN**, Goldwasser MA, Paszat LF, Saskin R, Urbach DR, Rabeneck L. Association of colonoscopy and death from colorectal cancer. *Ann Intern Med* 2009; **150**: 1-8 [PMID: 19075198]

29 **Brenner H**, Hoffmeister M, Arndt V, Stegmaier C, Altenhofen L, Haug U. Protection from right- and left-sided colorectal neoplasms after colonoscopy: population-based study. *J Natl Cancer Inst* 2010; **102**: 89-95 [PMID: 20042716 DOI: 10.1093/jnci/djp436]

30 **Singh H**, Nugent Z, Demers AA, Kliewer EV, Mahmud SM, Bernstein CN. The reduction in colorectal cancer mortality after colonoscopy varies by site of the cancer. *Gastroenterology* 2010; **139**: 1128-1137 [PMID: 20600026 DOI: 10.1053/j.gastro.2010.06.052]

31 **Sawhney MS**, Farrar WD, Gudiseva S, Nelson DB, Lederle FA, Rector TS, Bond JH. Microsatellite instability in interval colon cancers. *Gastroenterology* 2006; **131**: 1700-1705 [PMID: 17087932 DOI: 10.1053/j.gastro.2006.10.022]

32 **Arain MA**, Sawhney M, Sheikh S, Anway R, Thyagarajan B, Bond JH, Shaukat A. CIMP status of interval colon cancers: another piece to the puzzle. *Am J Gastroenterol* 2010; **105**: 1189-1195 [PMID: 20010923 DOI: 10.1038/ajg.2009.699]

33 **Cooper GS**, Xu F, Barnholtz Sloan JS, Schluchter MD, Koroukian SM. Prevalence and predictors of interval colorectal cancers in medicare beneficiaries. *Cancer* 2012; **118**: 3044-3052 [PMID: 21989586 DOI: 10.1002/cncr.26602]

34 **Tadepalli US**, Feihel D, Miller KM, Itzkowitz SH, Freedman JS, Kornacki S, Cohen LB, Bamji ND, Bodian CA, Aisenberg J. A morphologic analysis of sessile serrated polyps observed during routine colonoscopy (with video). *Gastrointest Endosc* 2011; **74**: 1360-1368 [PMID: 22018553 DOI: 10.1016/j.gie.2011.08.008]

35 **Kimura T**, Yamamoto E, Yamano HO, Suzuki H, Kamimae S, Nojima M, Sawada T, Ashida M, Yoshikawa K, Takagi R, Kato R, Harada T, Suzuki R, Maruyama R, Kai M, Imai K, Shinomura Y, Sugai T, Toyota M. A novel pit pattern identifies the precursor of colorectal cancer derived from sessile serrated adenoma. *Am J Gastroenterol* 2012; **107**: 460-469 [PMID: 22233696 DOI: 10.1038/ajg.2011.457]

36 **Ishigooka S**, Nomoto M, Obinata N, Oishi Y, Sato Y, Nakatsu S, Suzuki M, Ikeda Y, Maehata T, Kimura T, Watanabe Y, Nakajima T, Yamano HO, Yasuda H, Itoh F. Evaluation of magnifying colonoscopy in the diagnosis of serrated polyps. *World J Gastroenterol* 2012; **18**: 4308-4316 [PMID: 22969193 DOI: 10.3748/wjg.v18.i32.4308]

37 **Hazewinkel Y**, López-Cerón M, East JE, Rastogi A, Pellisé M, Nakajima T, van Eeden S, Tytgat KM, Fockens P, Dekker E. Endoscopic features of sessile serrated adenomas: validation by international experts using high-resolution white-light endoscopy and narrow-band imaging. *Gastrointest Endosc* 2013; **77**: 916-924 [PMID: 23433877 DOI: 10.1016/j.gie.2012.12.018]

38 **Yamashina T**, Takeuchi Y, Uedo N, Aoi K, Matsuura N, Nagai K, Matsui F, Ito T, Fujii M, Yamamoto S, Hanaoka N, Higashino K, Ishihara R, Tomita Y, Iishi H. Diagnostic features of sessile serrated adenoma/polyps on magnifying narrow band imaging: a prospective study of diagnostic accuracy. *J Gastroenterol Hepatol* 2015; **30**: 117-123 [PMID: 25088839 DOI: 10.1111/jgh.12688]

39 **Murakami T**, Sakamoto N, Ritsuno H, Shibuya T, Osada T, Mitomi H, Yao T, Watanabe S. Distinct endoscopic characteristics of sessile serrated adenoma/polyp with and without dysplasia/carcinoma. *Gastrointest Endosc* 2017; **85**: 590-600 [PMID: 27663716 DOI: 10.1016/j.gie.2016.09.018]

40 **Rex DK**, Ahnen DJ, Baron JA, Batts KP, Burke CA, Burt RW, Goldblum JR, Guillem JG, Kahi CJ, Kalady MF, O'Brien MJ, Odze RD, Ogino S, Parry S, Snover DC, Torlakovic EE, Wise PE, Young J, Church J. Serrated lesions of the colorectum: review and recommendations from an expert panel. *Am J Gastroenterol* 2012; **107**: 1315-1329; quiz 1314, 1330 [PMID: 22710576 DOI: 10.1038/ajg.2012.161]

41 **Lieberman DA**, Rex DK, Winawer SJ, Giardiello FM, Johnson DA, Levin TR. Guidelines for colonoscopy surveillance after screening and polypectomy: a consensus update by the US Multi-Society Task Force on Colorectal Cancer. *Gastroenterology* 2012; **143**: 844-857 [PMID: 22763141 DOI: 10.1053/j.gastro.2012.06.001]

42 **Morson BC**. Precancerous lesions of the colon and rectum. Classification and controversial issues. *JAMA* 1962; **179**: 316-321 [PMID: 14476361]

43 **Boparai KS**, van den Broek FJ, van Eeden S, Fockens P, Dekker E. Hyperplastic polyposis syndrome: a pilot study for the differentiation of polyps by using high-resolution endoscopy, autofluorescence imaging, and narrow-band imaging. *Gastrointest Endosc* 2009; **70**: 947-955 [PMID: 19595313 DOI: 10.1016/j.gie.2009.03.1172]

44 **Gurudu SR**, Heigh RI, De Petris G, Heigh EG, Leighton JA, Pasha SF, Malagon IB, Das A. Sessile serrated adenomas: demographic, endoscopic and pathological characteristics. *World J Gastroenterol* 2010; **16**: 3402-3405 [PMID: 20632442]

45 **Higuchi T**, Sugihara K, Jass JR. Demographic and pathological characteristics of serrated polyps of colorectum. *Histopathology* 2005;

47: 32-40 [PMID: 15982321 DOI: 10.1111/j.1365-2559.2005.02180.x]

46   **Tajiri H**, Niwa H. Proposal for a consensus terminology in endoscopy: how should different endoscopic imaging techniques be grouped and defined? *Endoscopy* 2008; **40**: 775-778 [PMID: 18698532 DOI: 10.1055/s-2008-1077507]

47   **Sano Y**, Horimatsu T, Fu KI, Katagiri A, Muto M, Ishikawa H. Magnifying observation of microvascular architecture of colorectal lesions using a narrow band imaging system. *Dig Endosc* 2006; **18**: S44-S51 [DOI: 10.1111/j.0915-5635.2006.00621.x]

48   **Chiu HM**, Chang CY, Chen CC, Lee YC, Wu MS, Lin JT, Shun CT, Wang HP. A prospective comparative study of narrow-band imaging, chromoendoscopy, and conventional colonoscopy in the diagnosis of colorectal neoplasia. *Gut* 2007; **56**: 373-379 [PMID: 17005766 DOI: 10.1136/gut.2006.099614]

49   **Uraoka T**, Sano Y, Saito Y, Saito H, Matsuda T, Yamamoto K. Narrow-band imaging for improving colorectal adenoma detection: appropriate system function settings are required. *Gut* 2009; **58**: 604-605 [PMID: 19299388 DOI: 10.1136/gut.2008.157164]

50   **Nakao Y**, Saito S, Ohya T, Aihara H, Arihiro S, Kato T, Ikegami M, Tajiri H. Endoscopic features of colorectal serrated lesions using image-enhanced endoscopy with pathological analysis. *Eur J Gastroenterol Hepatol* 2013; **25**: 981-988 [PMID: 23820237 DOI: 10.1097/MEG.0b013e3283614b2b]

51   **Yamada M**, Sakamoto T, Otake Y, Nakajima T, Kuchiba A, Taniguchi H, Sekine S, Kushima R, Ramberan H, Parra-Blanco A, Fujii T, Matsuda T, Saito Y. Investigating endoscopic features of sessile serrated adenomas/polyps by using narrow-band imaging with optical magnification. *Gastrointest Endosc* 2015; **82**: 108-117 [PMID: 25840928 DOI: 10.1016/j.gie.2014.12.037]

52   **Uraoka T**, Higashi R, Horii J, Harada K, Hori K, Okada H, Mizuno M, Tomoda J, Ohara N, Tanaka T, Chiu HM, Yahagi N, Yamamoto K. Prospective evaluation of endoscopic criteria characteristic of sessile serrated adenomas/polyps. *J Gastroenterol* 2015; **50**: 555-563 [PMID: 25270966 DOI: 10.1007/s00535-014-0999-y]

53   **Kudo S**, Hirota S, Nakajima T, Hosobe S, Kusaka H, Kobayashi T, Himori M, Yagyuu A. Colorectal tumours and pit pattern. *J Clin Pathol* 1994; **47**: 880-885 [PMID: 7962600]

54   **Kudo S**, Tamura S, Nakajima T, Yamano H, Kusaka H, Watanabe H. Diagnosis of colorectal tumorous lesions by magnifying endoscopy. *Gastrointest Endosc* 1996; **44**: 8-14 [PMID: 8836710]

55   **Kudo S**, Kashida H, Tamura T, Kogure E, Imai Y, Yamano H, Hart AR. Colonoscopic diagnosis and management of nonpolypoid

early colorectal cancer. *World J Surg* 2000; **24**: 1081-1090 [PMID: 11036286]

56   **Kudo S**, Rubio CA, Teixeira CR, Kashida H, Kogure E. Pit pattern in colorectal neoplasia: endoscopic magnifying view. *Endoscopy* 2001; **33**: 367-373 [PMID: 11315901 DOI: 10.1055/s-2004-826104]

57   **Parikh ND**, Chaptini L, Njei B, Laine L. Diagnosis of sessile serrated adenomas/polyps with image-enhanced endoscopy: a systematic review and meta-analysis. *Endoscopy* 2016; **48**: 731-739 [PMID: 27223636 DOI: 10.1055/s-0042-107592]

58   **Rex DK**, Clodfelter R, Rahmani F, Fatima H, James-Stevenson TN, Tang JC, Kim HN, McHenry L, Kahi CJ, Rogers NA, Helper DJ, Sagi SV, Kessler WR, Wo JM, Fischer M, Kwo PY. Narrow-band imaging versus white light for the detection of proximal colon serrated lesions: a randomized, controlled trial. *Gastrointest Endosc* 2016; **83**: 166-171 [PMID: 25952085 DOI: 10.1016/j.gie.2015.03.1915]

59   **Lash RH**, Genta RM, Schuler CM. Sessile serrated adenomas: prevalence of dysplasia and carcinoma in 2139 patients. *J Clin Pathol* 2010; **63**: 681-686 [PMID: 20547691 DOI: 10.1136/jcp.2010.075507]

60   **Chino A**, Yamamoto N, Kato Y, Morishige K, Ishikawa H, Kishihara T, Fujisaki J, Ishikawa Y, Tamegai Y, Igarashi M. The frequency of early colorectal cancer derived from sessile serrated adenoma/polyps among 1858 serrated polyps from a single institution. *Int J Colorectal Dis* 2016; **31**: 343-349 [PMID: 26510850 DOI: 10.1007/s00384-015-2416-2]

61   **Bouwens MW**, van Herwaarden YJ, Winkens B, Rondagh EJ, de Ridder R, Riedl RG, Driessen A, Dekker E, Masclee AA, Sanduleanu S. Endoscopic characterization of sessile serrated adenomas/polyps with and without dysplasia. *Endoscopy* 2014; **46**: 225-235 [PMID: 24573732 DOI: 10.1055/s-0034-1364936]

62   **Burgess NG**, Pellise M, Nanda KS, Hourigan LF, Zanati SA, Brown GJ, Singh R, Williams SJ, Raftopoulos SC, Ormonde D, Moss A, Byth K, P'Ng H, McLeod D, Bourke MJ. Clinical and endoscopic predictors of cytological dysplasia or cancer in a prospective multicentre study of large sessile serrated adenomas/polyps. *Gut* 2016; **65**: 437-446 [PMID: 25731869 DOI: 10.1136/gutjnl-2014-308603]

63   **Ban S**, Mitomi H, Horiguchi H, Sato H, Shimizu M. Adenocarcinoma arising in small sessile serrated adenoma/polyp (SSA/P) of the colon: clinicopathological study of eight lesions. *Pathol Int* 2014; **64**: 123-132 [PMID: 24698422 DOI: 10.1111/pin.12147]

ORIGINAL ARTICLE

Basic Study

Downregulation of Hes1 expression in experimental biliary atresia and its effects on bile duct structure

Rui-Zhong Zhang, Xin-Hao Zeng, Ze-Feng Lin, Ming-Fu, Yan-Lu Tong, Vincent CH Lui, Paul KH Tam, Jonathan R Lamb, Hui-Min Xia, Yan Chen

Rui-Zhong Zhang, Xin-Hao Zeng, Ze-Feng Lin, Ming-Fu, Yan-Lu Tong, Hui-Min Xia, Yan Chen, Department of Pediatric Surgery, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou 510623, Guangdong Province, China

Vincent CH Lui, Paul KH Tam, Yan Chen, Department of Surgery and Pathology, University of Hong Kong, Hong Kong, China

Jonathan R Lamb, Department of Life Sciences, Faculty of Natural Sciences, Imperial College London, London SW7 2AZ, United Kingdom

ORCID number: Rui-Zhong Zhang (0000-0002-4954-7192); Xin-Hao Zeng (0000-0002-3879-9810); Ze-Feng Lin (0000-0001-7532-9175); Ming-Fu (0000-0003-1400-0258); Yan-Lu Tong (0000-0003-0650-1562); Vincent CH Lui (0000-0002-1758-8854); Paul KH Tam (0000-0003-1596-4120); Jonathan R Lamb (0000-0002-3879-9810); Hui-Min Xia (0000-0002-0103-1672); Yan Chen (0000-0002-2354-230X).

Author contributions: Zhang RZ and Zeng XH contributed equally to this work; Zhang RZ, Lin ZF and Fu M performed the animal work; Lin ZF and Tong YL analyzed the immunohistochemistry; Lui VC, Tam PK, Lamb JR, Xia HM and Chen Y contributed to the study design, data collection, analysis, discussion and manuscript preparation.

Supported by the Science and Technology Project of Guangzhou, No. 201707010014; and the National Natural Science Foundation of China, No. 81600399 and No. 81671498.

Institutional review board statement: This study was reviewed and approved by the Institutional Review Board of Guangzhou Women and Children's Medical Center, Guangzhou, China, No. 2015090109.

Institutional animal care and use committee statement: All animal protocols were approved by the Institutional Animal Care and Use Committee of Guangzhou Medical University, Guangzhou, China, No. 2016-007.

Conflict-of-interest statement: The authors declare that there is no conflict of interest related to this study.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: In the manuscript, the ARRIVE Guidelines have been adopted.

Abstract

AIM

To analyze the expression and function of the notch

signaling target gene Hes1 in a rhesus rotavirus-induced mouse biliary atresia model.

METHODS
The morphologies of biliary epithelial cells in biliary atresia patients and in a mouse model were examined by immunohistochemical staining. Then, the differential expression of Notch signaling pathway-related molecules was investigated. Further, the effects of the siRNA-mediated inhibition of Hes1 expression were examined using a biliary epithelial cell 3D culture system.

RESULTS
Both immature (EpCAM[+]) and mature (CK19^{+}) biliary epithelial cells were detected in the livers of biliary atresia patients without a ductile structure and in the mouse model with a distorted bile duct structure. The hepatic expression of transcripts for most Notch signaling molecules were significantly reduced on day 7 but recovered to normal levels by day 14, except for the target molecule Hes1, which still exhibited lower mRNA and protein levels. Expression of the Hes1 transcriptional co-regulator, RBP-Jκ was also reduced. A 3D gel culture system promoted the maturation of immature biliary epithelial cells, with increased expression of CK19^{+} cells and the formation of a duct-like structure. The administration of Hes1 siRNA blocked this process. As a result, the cells remained in an immature state, and no duct-like structure was observed.

CONCLUSION
Our data indicated that Hes1 might contribute to the maturation and the cellular structure organization of biliary epithelial cells, which provides new insight into understanding the pathology of biliary atresia.

Key words: Biliary atresia; Rhesus rotavirus; Hes1; EpCAM; Epithelial cells 3D culture

Core tip: Similar immature biliary epithelial cells and distorted bile ductules were observed in biliary atresia patients and a mouse biliary atresia model. Investigation of the Notch signaling pathway target gene Hes1 showed that mRNA and protein expression was reduced in mouse liver, which was partially due to reduced expression of transcriptional co-regulator RBP-Jκ. In biliary epithelial cells 3D gel culture system, the administration of Hes1 siRNA blocked the maturation and duct-like structure formation process, which resulted in the cells still being in an immature state and no duct-like structure was observed.

Zhang RZ, Zeng XH, Lin ZF, Fu M, Tong YL, Lui VC, Tam PK, Lamb JR, Xia HM, Chen Y. Downregulation of Hes1 expression in experimental biliary atresia and its effects on bile duct structure. *World J Gastroenterol* 2018; 24(29): 3260-3272 Available from:

INTRODUCTION
Biliary atresia (BA) is the most common cause of end-stage liver disease in infants, and it has a poor prognosis with a high fatality rate. It is characterized by persistent jaundice and progressive cholestasis that develops within weeks of birth. These symptoms are caused by progressive extrahepatic and/or intrahepatic bile duct inflammation and tissue fibrosis in both the bile ducts and the liver. Although the symptoms can be partially ameliorated by Kasai surgery and extensive post-operative care, some of the patients still develop cirrhosis and liver failure due to the progressive destruction of intrahepatic bile ducts and liver fibrosis[1]. The incidence of BA in the USA is approximately 1:15000 live births, and the incidence is even higher in Asia at approximately 1:9600 live births[2,3]. Without liver transplants, the long-term survival rate of BA is between 13% and 50%[4]. Furthermore, the etiology of the disease is not fully understood. The birth defects and inflammation associated with viral infection and subsequent anti-viral immunity, which targets biliary epithelial cells (BEC), cause apoptosis and injury to the bile ducts. This results in a failure to reestablish the biliary system and progresses as tissue fibrosis, eventually leading to BA[5].

The Notch signaling pathway plays a key role in the development and differentiation of hepatic stem cells into BECs in order to establish the biliary system[6,7]. It is composed of a family of molecules, including Notch ligands, Notch receptors, DNA binding protein RBP-Jκ and effectors, such as HES, HEY, and HERP, which mediate the biological function of Notch signaling. Mutations in the Notch ligand Jagged1 (Jag1) or receptor Notch2 in humans results in Alagille syndrome (AGS), which is characterized by biliary dysplasia and cholestasis[8,9]. Similar findings have been observed in mice expressing null alleles of Jag1 or Notch2[10], which suggests a mechanism of functional conservation. Hes1 is a target gene of the Notch signaling pathway and is essential for the tubular formation in the biliary epithelium[11] and maintenance of the characteristics of the biliary epithelium[12]. However, the effect of altered Hes1 expression in BA has not been established.

Pathological observations have suggested that the blockade of the extrahepatic bile duct is the major change in both patients with BA and the animal model of BA, and that damage to the intrahepatic bile duct further enhances bile accumulation in the liver, which promotes liver fibrosis. The number of intrahepatic biliary epithelial cells (IBECs) might differ from patient to patient, but immature cell status and malformed ductular structures were commonly observed in patient liver samples[13]. Epithelial cell adhesion molecule (EpCAM) is expressed

in the embryonic stage of liver development[14], and some studies have used it as an immature epithelial cell marker for cell isolation[15]. CK19^{+} represents a biliary epithelial cell lineage marker. It is only weakly expressed in hepatocytes, whereas it is highly expressed in mature BECs[16,17]. Using these two markers in this study, the presence of BECs was examined in rhesus rotavirus (RRV)-inoculated neonatal BA mice. The expression levels of components of the Notch signaling pathway, notably Hes1, were detected, and the effects of Hes1 loss of function on BEC morphological changes were evaluated in 3D cell culture.

MATERIALS AND METHODS

Reagents and antibodies

The rhesus rotavirus strain MMU 18006 (RRV) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, United States). The virus was amplified in MA104 cells, and the virus quantification was measured by a plaque assay method, as described previously[18]. The antibodies used for immunohistochemical analyses of mouse tissue sections were rat anti-cytokeratin 19 (CK19, clone TROMA Ⅲ) and rat anti-EpCAM (clone G8.8), both of which were purchased from DSHB (Developmental Studies Hybridoma Bank, Iowa City, IA, United States). Rabbit anti-human Hes1 and rabbit anti-human RBP-Jκ were obtained from Cell Signaling Technology (Cell Signaling Technology Danvers, MA, United States). For human tissue section staining, rabbit anti-human CK19 and rabbit anti-human EpCAM were both obtained from Santa Cruz (Santa Cruz Biotechnology, Inc. Dallas, TX, United States). The RNA extraction kit and the reverse transcription reagents were purchased from Invitrogen (Life Technologies Limited, NT, Hong Kong), and Super Real Pre-Mix SYBR green was purchased from Tiangen [Tiangen Biotech (Beijing) Co., Ltd., Beijing, China].

Human samples and pathological analysis

Clinical liver tissue samples from BA patients (*n* = 20) and disease controls [with choledochal cyst (CC), *n* = 3] were obtained from the Pathology Department of Guangzhou Women and Children's Medical Center. The data were analyzed anonymously. The diagnosis was established by both pathologists and physicians based on clinical observation during operations and a laboratory-based pathological analysis. Tissue fibrosis was demonstrated by Masson tricolor staining and Sirius Red staining (data not shown). The ages of the patients included in the study ranged from two to five months old. The experimental protocols were approved by the Medical Ethics Committee of Guangzhou Women and Children's Medical Center (2015090109).

Electron microscopy analysis

The tissues were fixed in neutral glutaraldehyde and osmic acid. After dehydration, the tissues were embedded in resin. Tissue blocks were first sectioned at 1-2 microns with glass knives using an EM UC7 ultramicrotome (Leica, Buffalo Grove, IL, United States), and a reference was used to trim the blocks for thin sectioning. The appropriate blocks were then thinly sectioned at 50-70 nm. After drying on filter paper, the sections were stained with uranyl acetate and lead citrate for contrast. After drying, the grids were then viewed on a JEM-1400 microscope (JEOL USA, Inc., Peabody, MA, United States).

Infection of neonatal mice with rhesus rotavirus

Day 12.5 pregnant Balb/c mice aged between 10 and 12 wk were purchased from Guangdong Animal Experimental Centre, maintained under specific pathogen-free conditions and housed in a room with a 12-h dark-light cycle. All of the animal protocols were approved by The Institutional Animal Care and Use Committee of Guangzhou Medical University (2016-007).

To establish an experimental model of BA, neonatal mice were intraperitoneally injected within 24 h of birth with 20 μL of either 4×10^4 PFU/mL RRV or MA104 cell culture supernatant as a control. Infected mice that died within the first two days or that were not fed by their mothers were excluded from further analysis. All appropriate measures were taken to minimize the pain and discomfort of the mice. The mice were weighed and examined daily, and, in general, the development of icterus of the skin not covered with fur and acholic stools were generally observed on days five to six after the injection, indicating a successful induction of BA. The mice were sacrificed by an overdose of pentobarbitone (200 mg/kg, i.p.). To obtain tissue samples, the animals were dissected under a microscope (Nikon SMZ1000), and the gross appearances of the livers and bile ducts were recorded. The tissues were harvested and stored at -80 ℃ for RNA/protein isolation and in 10% formalin for histological sample preparation.

Histological and immunohistochemical analysis

Mouse and human liver samples were fixed and paraffin-embedded for immunostaining. Then, 4 μm thick tissue sections were rehydrated and then stained with hematoxylin and eosin (HE) for histological analysis. For immuno-histochemistry, antigen retrieval was performed in citrate buffer [10 mmol/L, 0.01% Tween20, pH 6.0 for EpCAM or Tris-EDTA buffer (10 mmol/L Tris base, 1 mmol/L EDTA solution, pH 9.0 for CK19)], and for the removal of endogenous peroxidase, the samples were treated with 3% hydrogen peroxide. The sections were then incubated overnight at 4 ℃ in blocking solution containing primary antibodies. Blocking solution without primary antibodies only was used as a control. After undergoing appropriate secondary antibody incubation, the immunostaining was completed using a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, United States) and liquid 3,3'-diaminobenzidine (Dako). The results were analyzed using a Nikon microscope, and images were captured with NIS-Elements F4.0.

Table 1 Primers used in the experiments

Genes	5'→3'	Sequences
Mouse Jag1	Forward	TTCTCACTCAGGCATGATAAACC
	Reverse	CATCTCTGGGACGACAGAACT
Mouse Dll1	Forward	CCCATCCGATTCCCCTTCG
	Reverse	GGTTTTCTGTTGCGAGGTCATC
Mouse Notch1	Forward	CCCTTGCTCTGCCTAACGC
	Reverse	GGAGTCCTGGCATCGTTGG
Mouse Notch2	Forward	CTGTGAGCGGAATATCGACGA
	Reverse	ATAGCCTCCGTTTCGGTTGG
Mouse Hes1	Forward	CCAGCCAGTGTCAACACGA
	Reverse	AATGCCGGGAGCTATCTTTCT
Mouse β-actin	Forward	AAACTGGAACGGTGAAGGTG
	Reverse	AGTGGGGTGGCTTTTAGGAT
Human Hes1	Forward	TCAACACGACACCGGATAAAC
	Reverse	GCCGCGAGCTATCTTTCTTCA
Human β-actin	Forward	TTAGTTGCGTTACACCCTTTCTTGACA
	Reverse	CTGTCACCTTCACCGTTCCAGTTTT

Gene expression profiling using quantitative PCR

The expression levels of Notch signaling-related molecules in the livers (harvested at 7 d and 14 d) of RRV-inoculated and saline control mice were determined using qPCR. The total RNA was extracted from a portion of each liver using TRIzol (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions, and the extracts were treated with DNAse. cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, United States) according to the manufacturer's instructions. The primers used in the experiments are shown in Table 1, and PCR was performed with a C1000 Thermal Cycler (Bio-Rad Laboratories Co., Ltd., CA, United States).

Protein expression analysis

Protein expression levels were analyzed by Western blotting according to standard protocols, and photographs were captured using a Bio-Rad molecular Imager® Gel Doc™ XR+ imaging system with Image Lab™ software. The primary antibodies against Hes1 (1:1000), RBP-Jκ (1:1000), and β-actin (1:3000) and the appropriate secondary antibodies coupled with horseradish peroxidase (Bio-Rad, Munchen, Germany) were used to label the protein bands.

3D biliary epithelial cell culture

IBECs were purchased from ScienCell (Cat. Number 5100). Epithelial Cell Medium (EpiCM, ScienCell, Carlsbad, CA, United States. 4101) and additional Epithelial Cell Growth Supplement (EpiCGS, ScienCell 4152) were used for the maintenance and growth of IBECs. The medium used for the differentiation of cells was based on Kubota's Hepatoblast Growth Medium (PhoenixSongs Biologicals, Inc. Branford, CT, United States. 06405 11002-250). For IBEC differentiation, the medium was supplemented with HGF (10 ng/mL) and VEGF (20 ng/mL) (PeproTech Asia, Rehovot, Israel). The identification of the cell type was performed by immunofluorescence staining using the following 3 antibodies: AFP (Abcam, GR201677-8, 1:200) for hepatocytes and CK19 (Santa Cruz Biotechnology, K2613, 1:200) and γ-GT (Abcam, GR304453-1, 1:300) for BECs. Goat anti-mouse Alexa Fluor® 488 secondary antibody (Life Technologies, 1613346) and DAPI (KeyGen Biotech. Co., Ltd., Nanjing, China. KGR0001) were used to visualize antibody staining and the nuclei of the cells.

For the formation of duct-like structures of IBECs in three-dimensional culture, a 4:6 (v/v) gel mixture of BD Matrigel Matrix Growth Factor Reduced High Concentration (BD, 356230) and collagen Type I Rat Tail (CORNING, 354236) was used. Cells (4×10^4) were seeded on the gel using a 15-well μ-Plate Angiogenesis plate (ibidi GmbH, Martinsried, Germany, 81506) and cultured in Kubota's hepatoblast differentiation medium for seven days. The immunofluorescence staining described above was used to show the structure and differentiation statuses.

Transfection assay

To study the interruption of gene expression, such as interruption of the Notch signaling pathway target molecule Hes1, in the IBEC 3D cell culture, Hes1 siRNA RNA and control siRNA purchased from Santa Cruz Biotechnology, Inc., were used in the cell culture and transfected into cells using Lipofectamine® RNAiMAX Reagent (Life Technologies, 13778) following the manufacturer's instructions.

Statistical analysis

The data are presented as the mean ± SEM when repeated measurements were used and as the mean ± SD when individual values were used. The statistical analysis was performed by the Mann-Whitney test when two sets of data were compared and by the Kruskal-Wallis test when more than three sets of data were compared. The statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, United States) with P values < 0.05 taken as
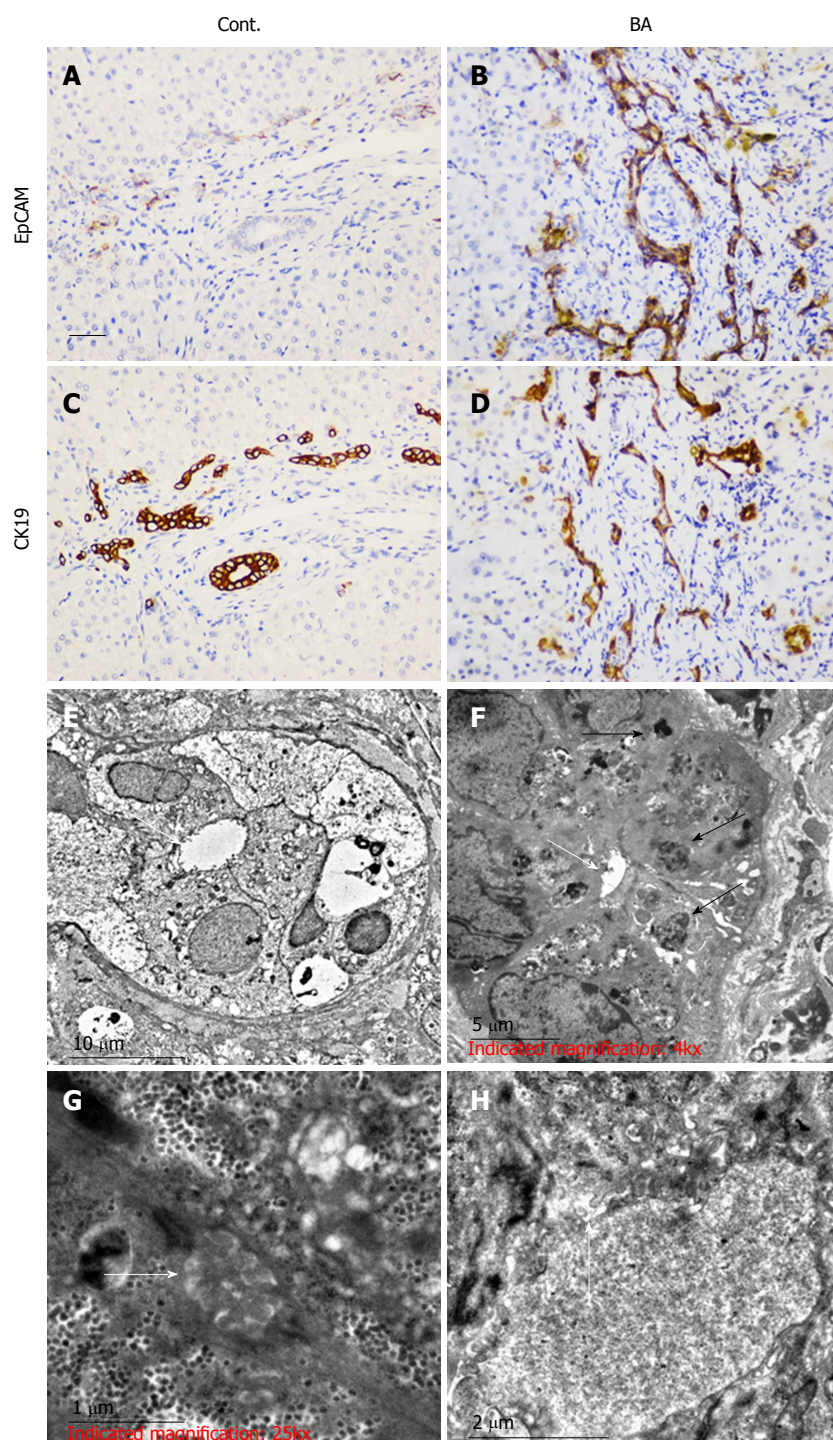
Figure 1 Expression levels of EpCAM and CK19 in neonatal liver biopsies and electron microscopy analysis of hepatic ultrastructure. To test the biliary epithelial marker expression levels, immunohistochemical staining was performed on disease control samples (Cont., choledochal cyst, A and C) and biliary atresia patient samples (BA, B and D). For the colocalization study of EpCAM and CK19, adjacent tissue sections were used. The scale bar is shown as 50 μm. For the EM analysis, liver tissues from the Cont. (E and F) and BA patients (G and H) were sectioned at 50-70 nm and stained with uranyl acetate and lead citrate. Photographs of the bile capillary (E and G) and microvilli (F and H) were captured and presented. The scale bar is also shown in the photographs. *n* = 5 each for the CC and BA groups.

the level of significance.

RESULTS

Increase in EpCAM immunopositive cells in the liver tissue of BA patients and the study of the ultrastructure of cells

We used adjacent, age-matched sections for studying immature BECs (EpCAM[+]) and immunohistochemical staining of the BEC marker (CK19^{+}). Our results showed that compared with choledochal cysts as a control (Cont., Figure 1A and C) there were highly increased numbers of EpCAM^{+} cells in BA (Figure 1B and D). Most EpCAM^{+} cells were also CK19^{+}, indicating that EpCAM is expressed in BECs. In choledochal cysts, disappearance

of the EpCAM[+] marker indicated the mature status of the cells. Additionally, the bile ductules could be easily observed in choledochal cysts but not in BA.

The ultrastructural changes in the morphology of the bile ducts in BA patients were compared to those in CC patients. The results revealed a similar hepatic cellular ultrastructure appearance in both cases. Mild swelling of liver cells was observed with no obvious abnormalities in the nuclei, rough endoplasmic reticulum, or smooth endoplasmic reticulum, apart from slight hyperplasia. However, in the portal triad, abnormal narrowing of the bile capillary was observed together with irregular sediments of bilirubin particles in the BA group (Figure 1F) in contrast to the CC group (Figure 1E). Fewer microvilli and less bile salt deposition were found in the BA patient tissue sections (Figure 1H) than in the CC patient tissue sections (Figure 1G). Taken together, these data suggest that BECs in BA patients are not fully mature, which might affect bile transport.

Optimization of the dose of rhesus rotavirus for the induction of BA in mice

Viral infection is proposed to be a causative factor in the development of BA, and the RRV-induced BA mouse model is used to mimic the pathophysiological processes in human BA. However, high doses of virus (1×10^6 PFU/mL) often cause the early death of mice at days 5-7 after viral inoculation. To reduce mortality, we used a smaller dose of RRV (4×10^4 PFU/mL) to induce BA. After viral inoculation, the mice exhibited BA syndrome with jaundice (Figure 2A) and extrahepatic bile duct occlusion (Figure 2B). The survival rate with this dosage was 63.03% and significantly differed from that of the control group (supernatant without virus infection) (Figure 2C; $P < 0.05$). Changes in weight loss were significant, with the control group exhibiting a 10.0 g $\pm$ 0.25 g change ($n = 15$) but the RRV-treated group only exhibiting a 5.6 g $\pm$ 1.3 g change ($n = 26$) (Figure 2D; $P < 0.001$). After dissection and tissue sectioning of the livers of the BA mice, HE staining revealed that, in contrast to the normal control group (Figure 2E), a disappearance of the common bile duct was observed in the BA group (Figure 2F). At the portal triads, enlarged cystic ducts and inflammatory cell accumulation were also present at day 14 after RRV inoculation, in contrast to normal control mice (Figure 2H *vs* Figure 2G).

Presence of EpCAM[+] and CK19^{+} bile ducts in RRV-inoculated BA mice

To identify the bile ducts, immunohistochemical staining was performed for both EpCAM and CK19 at days 7 and 14 after RRV inoculation, and RRV-inoculated mice were compared with control mice. As shown in Figure 3, EpCAM[+] bile ducts were noted at both days 7 and 14 in the control mice (Figure 3A and 3C), but few EpCAM^{+} cells were found in the RRV-inoculated mouse BA livers at day 7 (Figure 3B). At day 14, more EpCAM^{+} cells were detected, even in the areas of the portal tracts that

were infiltrated with inflammatory cells. However, none of these cells were organized into ductule-like structures (Figure 3D). Similar to EpCAM, CK19^{+} ductules were observed in the control livers at days 7 and 14, although the signal was slightly weaker on day 7 (Figure 3E and G). However, in BA mice, CK19^{+} cells were found in the portal areas, but no ductule structures were observed (Figure 3F and H). This observation suggests the presence of BECs, especially at day 14 after RRV inoculation. However, their development, especially in the formation of the bile duct process, was interrupted.

Expression of Notch signaling pathway components in the liver tissues of RRV-inoculated BA mice

The Notch signaling pathway plays a key role in bile duct system development. To examine its function in this model, qPCR was employed to quantify the expression levels of key genes in the Notch signaling pathway. The results showed that at day 7, there was a marked reduction in the expression levels of Notch ligands [Jag1 and Delta-like (Dll1)], Notch receptors (Notch1 and Notch2), and the Notch target gene Hes1 in the BA liver tissues compared with control liver tissues (Figure 4A, $P < 0.05$ for Jag1 and Notch1, $P < 0.01$ for Hes1 compared with the control). At day 14, no obvious differences in the expression levels of Jag1, Dll1, Notch1 and Notch2 were observed between the control and BA groups, and a slight increase in the expression of Notch2 was even found, although not statistically significant ($P = 0.1434$). However, the levels of Hes1 transcripts remained significantly reduced (10-fold compared with the control; $P < 0.001$; Figure 4B). The protein expression levels were then examined using Western blotting, which yielded similar results, namely, that the concentration of Hes1 protein in the mouse BA group was significantly lower on both day 7 and day 14 after RRV inoculation than that in the control group (Figure 4C). These data suggest that BA in RRV-inoculated mice may be caused, at least partially, by the downregulation of Hes1 expression.

Downregulation of RBP-J$_\kappa$ protein in the liver tissues of BA mice

As we observed reduced expression of Hes1, we then chose to investigate the expression levels of the regulators of Hes1. We determined the expression of the cotranscriptional factor RBP-J$_\kappa$ by Western blotting, and the results demonstrated that there was a minimal expression of RBP-J$_\kappa$ at both day 7 and day 14 in the BA mice compared to controls (Figure 4C). This finding suggests that downregulation of RBP-J$_\kappa$ may be the cause of the reduced expression of Hes1 and may subsequently hinder normal bile duct development.

Hes1 siRNA in the development of duct-like structures in 3D cell culture

The function of Hes1 in bile duct development was evaluated using a Hes1 siRNA transfection assay in a BEC
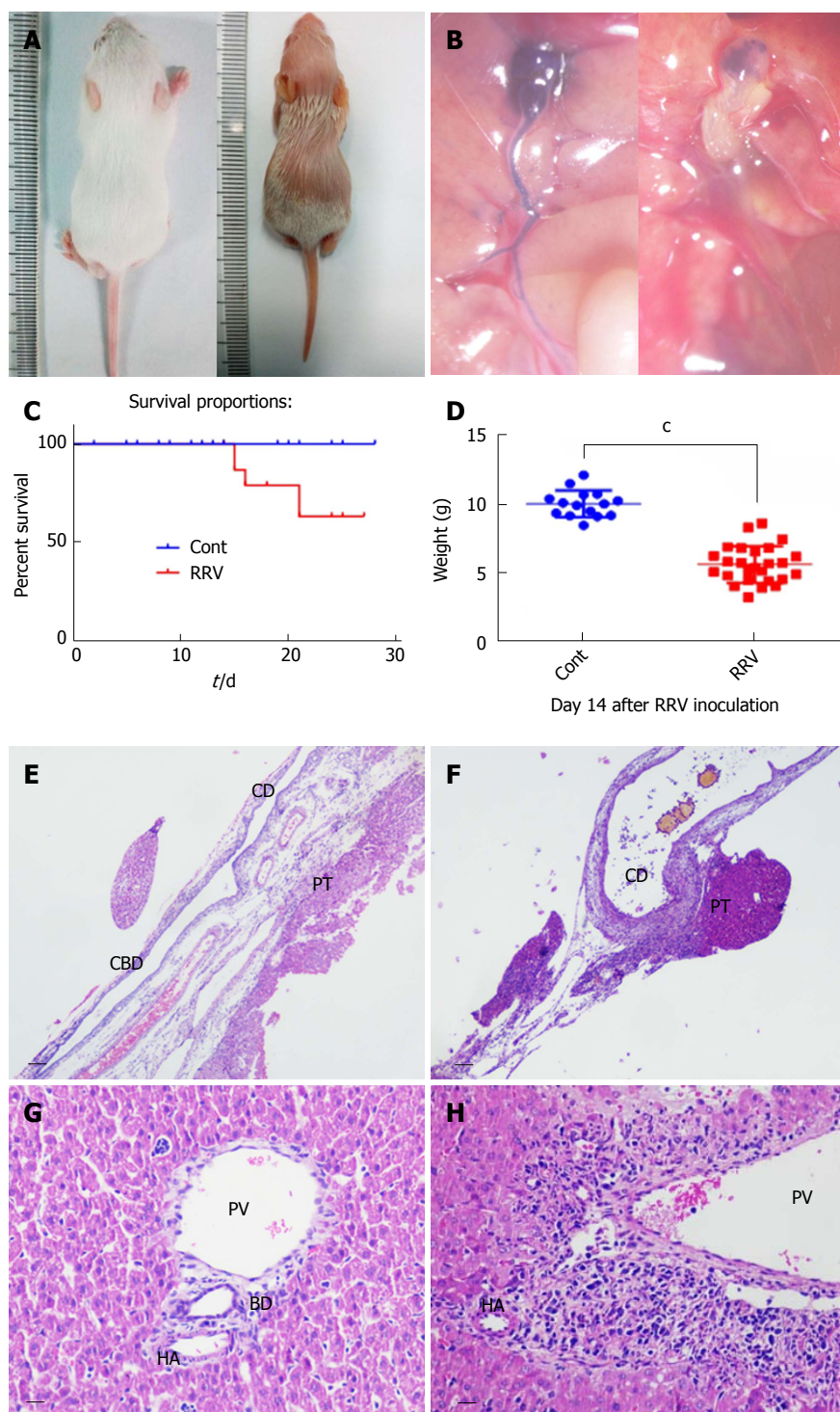
Figure 2 Biliary atresia in neonatal mice with a low dose of rhesus rotavirus inoculation. To study the expression levels of the components of the Notch signaling pathway in the biliary atresia (BA) animal model, a lower dose of 20 μL of rhesus rotavirus (RRV) with a titer of 4×10^4 PFU/mL was used. The morphology and extrahepatic block were photographed (A and B). The survival rate was measured at 21 d after viral inoculation (C). BA syndrome was observed between days 5 and 6. The body weights of the mice were measured at day 14 and compared with that of the control group (injected with culture supernatant but no virus) (D) (P < 0.001, n = 15 in the Cont. group, and n = 26 in the RRV group). After dissection, the portal triad (E and F) and liver tissue at day 14 (G and H) were sectioned, and histological analysis was performed. The scale bar is shown as 0.2 mm in the portal triad sections and as 50 μm in the liver tissue sections. CD: Cystic duct; CBD: Common bile duct; PT: Portal triad; PV: Portal vein; HA: Hepatic artery; BD: Bile duct.

culture. When BECs were cultured in growth medium, immunostaining showed that the cells expressed positive signals for CK19 and γ-GT but also a positive signal for AFP, which suggests the immature status of the cells (Figure 5, N. Medium). However, when the cells were

cultured in matrix gel + collagen gel (the mixed gel), the cells showed positive signals only for the BEC markers CK19 and γ-GT (Figure 5, Cont.), which suggested that the maturation process was underway. The formation of cells into duct-like structures was also observed
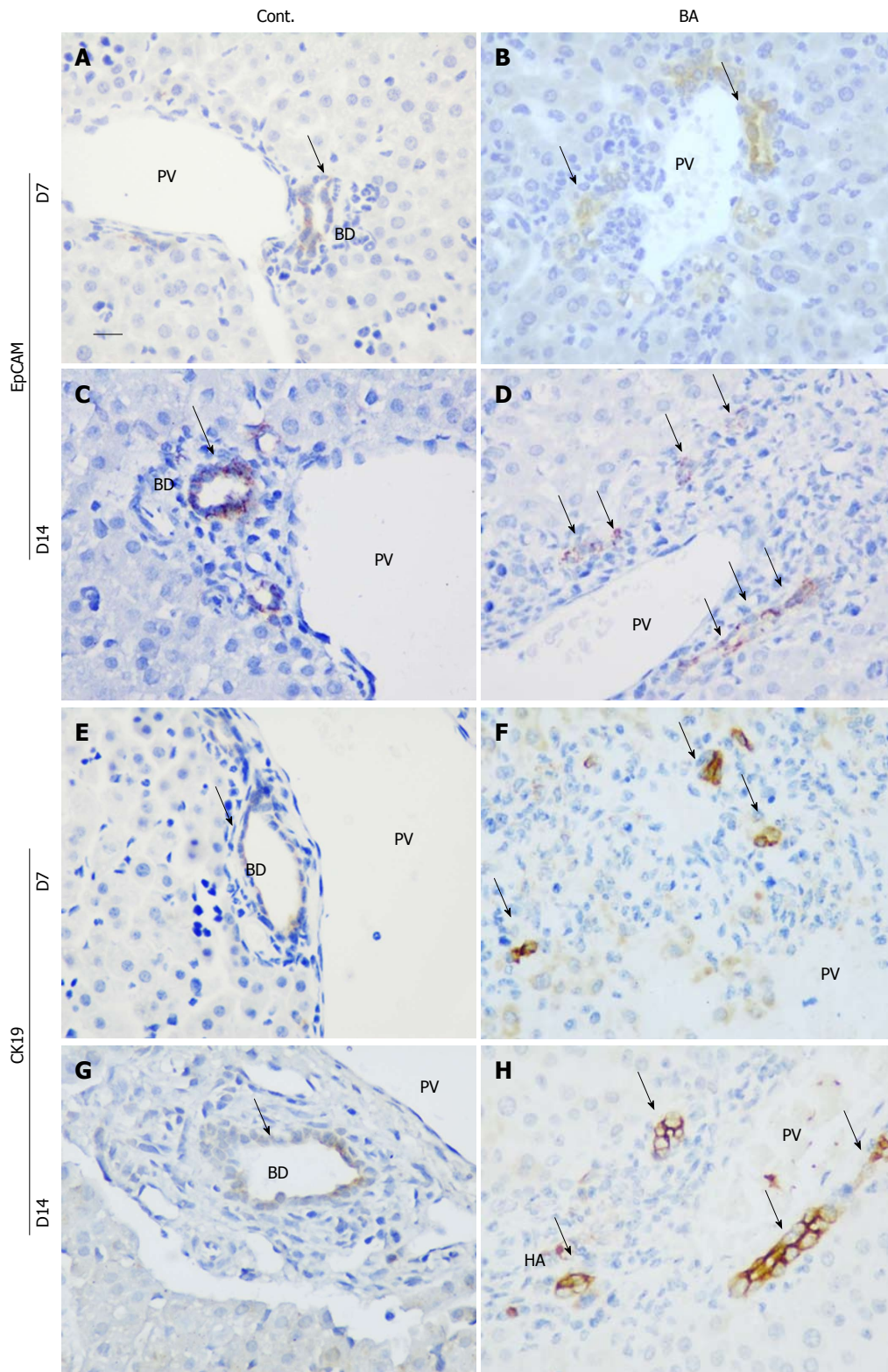
Figure 3 Expression levels of EpCAM and CK19 in the liver and ultrastructure of biliary epithelial cells. The expression levels of EpCAM and CK19 were analyzed using immunohistochemical staining with antibodies. The tissue sections were collected at day 7 (A, B, E and F) and day 14 (C, D, G and H). After they were dewaxed, the sections were stained with EpCAM and CK19 antibodies, and a comparison of the mice in the control group (Cont.) and those in the rhesus rotavirus inoculation group was performed. The scale bar shown is 100 μm. The black arrow indicates the positive signal. BA: Biliary atresia; CD: Cystic duct; CBD: Common bile duct; PT: Portal triad; PV: Portal vein; HA: Hepatic artery; BD: Bile duct.

after seven days in cell culture (Figure 5, Cont.). An enhanced density of duct-like structures was observed with prolonged culture time. These data indicated that

the culture conditions promoted the maturation of cells. The low toxicity of the transfection reagent for duct-like structure formation was tested, and the results showed
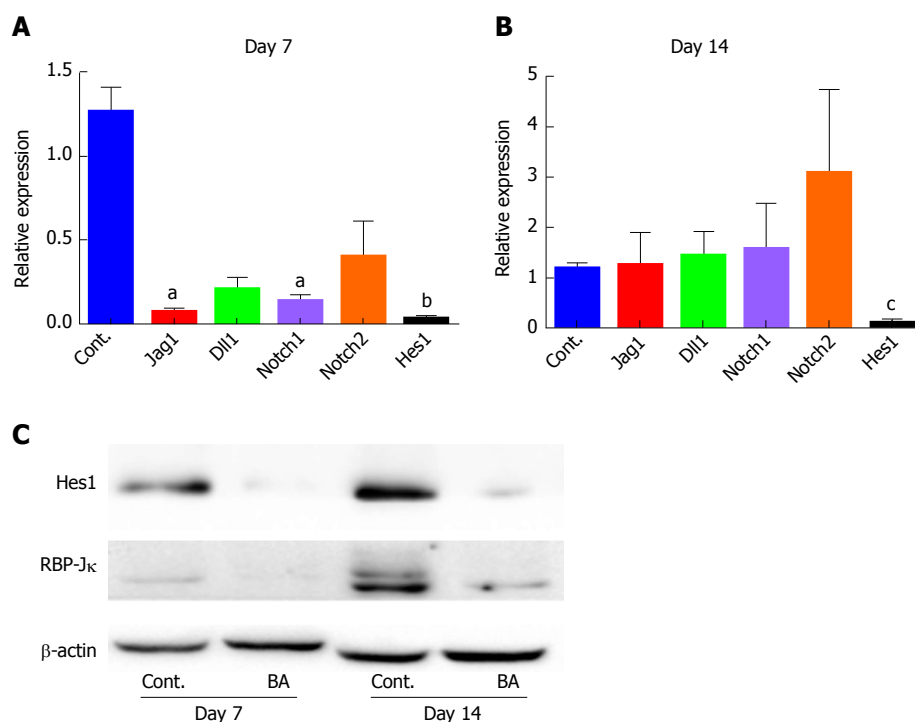
Figure 4 Expression levels of Notch signaling components in the liver of rhesus rotavirus-inoculated mice. The mRNA levels of Notch signaling components, including Jag1, Dll1, Notch1, Notch2 and Hes1, were detected using quantitative real-time PCR with appropriate primers at day 7 and day 14 after RRV inoculation. A: The relative expression levels compared with the normal control are shown for day 7 ([a]$P < 0.05$; [b]$P < 0.01$; $n = 6$ in the control group and $n = 10$ in the RRV group); B: day 14 ([c]$P < 0.001$); C: The levels of the Hes1 protein and the expression of the transcriptional coregulator RBP-Jκ were detected by Western blot ($n = 2$ or 3 in each group; the proteins were mixed and loaded).

that there was no obvious difference in duct formation in the transfection reagent group (Figure 5, Lipo) compared with the control group. However, Hes1 siRNA transfection induced an obvious difference compared with the irrelevant siRNA control (Figure 5, Hes1 siRNA *vs* Cont. siRNA), as no duct-like structures were observed in Hes1 siRNA-transfected cells, and these cells were kept in an immature state according to AFP[+] staining.

DISCUSSION

EpCAM is a cell surface-expressed transmembrane molecule that is present not only on epithelial cells but also on a variety of cells, including stem cells and cancer stem cells[19]. Using a BEC marker for colocalization, CK19, we noted that the number of EpCAM^{+} cells was increased in BA patients but not in CC control patients, suggesting active proliferation of BECs in BA. Furthermore, CK19 has been reported as a marker for ductal reactivity in tumor studies[20,21]. EpCAM expression in epithelial cells has been linked to the epithelial-mesenchymal transition (EMT)[22], but its role in this process remains controversial[23]. Both EMT and the ductal reaction are relevant to the development of tissue fibrosis[24], although the associated mechanisms have not been fully defined[25]. Elucidating EpCAM expression and its regulation in BA patients and/or experimental models of BA might provide new information regarding

the relationship of the ductal reaction with tissue fibrosis, especially as some previous studies have already reported the occurrence of EMT in BA patients[26-28].

We found that EpCAM[+] cell number was closely correlated with the expression of the Notch signaling target gene Hes1 and that Hes1 expression was increased in the nuclei of BECs in BA patients and RRV-inoculated mice. The expression of Hes1, as a nuclear regulatory protein, has been well-studied in biliary tract development, and as such, the Hes1-null mice showed a relatively normal ductal plate consisting of cytokeratin-positive and DBA-positive cholangiocyte precursors. However, by postnatal day 0, these mice presented with gallbladder agenesis and severe hypoplasia of the extrahepatic bile ducts[12], suggesting immature differentiation of BECs. In accordance with previous observations, our data of the 3D BEC cell culture also demonstrate that low levels of Hes1 prevented the formation of duct-like structures. Additionally, the increase in the expression levels of the mature BEC markers γ-GT and CK19 and the reduction in the expression of AFP are further evidence that Hes1 functions in the maturation and structural reorganization of these cells. We sought to examine the expression of Hes1 in human BA samples. Immunohistochemistry staining revealed heterogeneity in the results, even among the same tissue samples (data not shown). Thus, the Hes1 effect in BA might be related to different stages (inflammation or fibrogenesis) of the
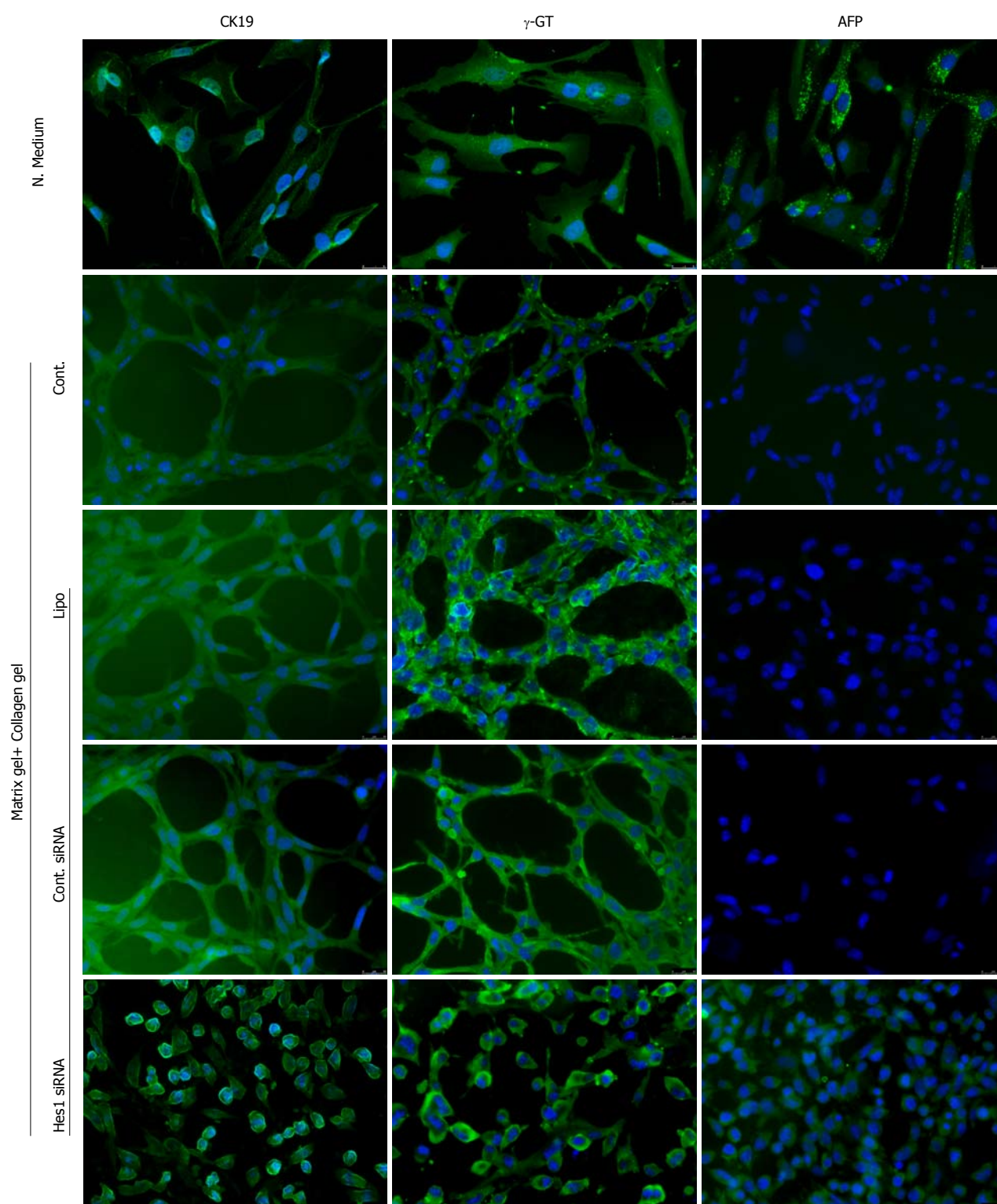
Figure 5 Hes1 siRNA in 3D biliary epithelial cell culture. Biliary epithelial cells (BECs) were cultured in either normal growth medium (N. medium) or in matrix + collagen mixed gel (detailed components are given in the Materials and Methods section). The cells were cultured without a transfection reagent (Cont.), with Lipofectamine (Lipo) only, with irrelevant control siRNA (Cont. siRNA) or with Hes1 siRNA for seven days, then fixed and stained with anti-CK19, γ-GT and AFP antibodies, and finally counter-stained with Alexa Fluor®488. Photographs of the cells were taken with a Leica DMi8 inverted fluorescence microscope, and the groups were then compared. The experiment was duplicated, and one set of representative results is shown here.

disease, as most of the BA patients in the clinic were in the stage of tissue fibrosis or later stages of the disease. Recruiting earlier-stage patients in whom inflammation is still progressing will help to clarify the alterations in Hes1 expression in BA patients.

The expression of Hes1 is under the control of coregulatory factors, particularly NICD and RBP-Jκ. NICD is a component of the Notch signaling pathway, and in our

study, its expression was relatively normal, although some intracellular signaling pathway components, such as gamma secretase, might still affect the levels of NICD[29]. Here, we provide evidence of a clear decline in the expression of RBP-Jκ, which might be the cause of reduced Hes1. A similar phenomenon was observed in RBP-Jκ KO mice. In those animals, the mature ducts were significantly reduced with respect to similarly treated wild-type mice[30]. It has been reported that RBP-Jκ expression is modulated by many regulatory factors, including viral infection[31,32]. In Epstein-Barr virus (EBV) infection, the nuclear antigens (EBNA) 3A and 3C can compete with EBNA 2 in binding with RBP-Jκ, thus inhibiting RBP-Jκ function. This diminishes the functional activity of Notch signaling by reducing the expression of Hes1[33]. However, whether or not there is any similarity between the EBV and RRV components in Hes1 expression remains to be investigated. At the end of the study, a high dose of Pentobarbital was administered to the mice, and then samples were collected for analysis. It has been shown that Pentobarbital affects bile secretion[34], however, as we used a control group, and the comparative results showed a difference between the treatment and control groups, the effect of Pentobarbital might not have directly influenced the results. Further research with mouse sacrifice by dislocation of the cervical spine will help to clarify this point.

The results of this study suggest that the reduced Hes1 expression may have contributed to reformation of the functional bile duct, but other factors cannot be excluded, such as inflammation or viral infection. The construction or adaptation of conditional knockout mice with targeted Hes1 knockdown on day 7, together with the BA mouse model with Hes1 overexpression, might help to confirm that Hes1 is the only contributing factor in this BA mouse model. The results of these experiments will provide more information regarding whether Hes1 overexpression has any therapeutic effect in the treatment of BA.

The mouse model used in this study represents acute viral infection-induced biliary atresia, in which tissue fibrosis is absent. The direct comparison of the mouse data with the patient data is not appropriate, as in patients, substantial tissue fibrosis is often observed at the later stage. Therefore, the development of a chronic BA mouse model will better mimic the condition observed in BA patients, allowing for a more accurate comparison of Hes1 data.

In conclusion, our study demonstrated that EpCAM+ cells are increased in the liver tissue of BA patients and the BA mouse model. The experimental results presented here suggest that the Notch signaling effector gene Hes1 might contribute to the disease processes in BA and that downregulation of RBP-Jκ might account for the pathophysiological changes in the bile ducts that were observed in the BA mouse model. The 3D BEC culture further confirmed the effect of Hes1 on the maturation and the duct-like structural formation of cells, which helps to further explain the dysregulation of structure formation in BA patients.

ARTICLE HIGHLIGHTS

Research background
An increased number of immature biliary epithelial cells and distorted bile ductules were observed in biliary atresia (BA), but the causes of these changes are unknown. The Notch signaling pathway is related to the development and differentiation of biliary epithelial cells. The target gene Hes1 is essential for tubular formation and maintenance. However, the effect of altered Hes1 expression in biliary atresia has not been established.

Research motivation
Notch signaling is one of the main pathways involved in bile duct development. However, its function in BA is not well known. Analysis of Notch signaling molecules using an established BA animal model, and 3D cell culture system might provide novel insights into the pathogenesis of BA.

Research objectives
The expression of Notch signaling pathway-related molecules was detected in a BA mouse model. The function of Hes1 downregulation was further examined using a 3D cell culture system. The results of this study can be expanded upon in future research of human BA patients by examining Hes1 expression and its relationship with BA pathogenesis.

Research methods
Immature biliary epithelial cells and bile duct structure distortion were examined in BA patients and in a BA mouse model. The expression of Notch signaling pathway-related molecules was detected in the mouse model by qPCR, and the expression of Hes1 and its gene regulatory protein was further confirmed by Western blotting. Finally, in 3D cell culture, the effects of Hes1 inhibition induced by siRNA transfection on duct-like structure formation were observed.

Research results
The results revealed the presence of immature biliary epithelial cells and distorted structures in both the BA patients and animal model. The downregulation of Hes1 expression, together with its transcriptional co-regulator RBP-Jκ, was observed in the BA mouse model. The siRNA-mediated inhibition of Hes1 completely blocked duct-like structure formation in the 3D cell culture system. However, Hes1 expression in BA patients must be further evaluated to confirm its function in the disease process.

Research conclusions
In conclusion, the results of the current study indicate that the immature biliary epithelial cells and defective duct-like structure formation in BA might be partly related to downregulation of the expression of the Notch signaling target gene Hes1. The use of a 3D epithelial cell culture system might help to identify other potential molecules, including those involved in epithelial cell maturation and duct-like structure formation.

Research perspectives
The potential effects of Hes1 observed in the BA mouse model and cell culture involving biliary epithelial cell maturation and duct-like structure formation suggest that Hes1 might contribute to the pathogenesis of BA. However, further examination of BA patient samples is necessary to better understand the role of Hes1 in the BA disease process.

ACKNOWLEDGMENTS

Kong for assisting with the statistical analysis.

REFERENCES

1   **Hartley JL**, Davenport M, Kelly DA. Biliary atresia. *Lancet* 2009; **374**: 1704-1713 [PMID: 19914515 DOI: 10.1016/S0140-6736(09)60946-6]

2   **Yoon PW**, Bresee JS, Olney RS, James LM, Khoury MJ. Epidemiology of biliary atresia: a population-based study. *Pediatrics* 1997; **99**: 376-382 [PMID: 9041292 DOI: 10.1542/peds.99.3.376]

3   **Davenport M**. Biliary atresia. *Semin Pediatr Surg* 2005; **14**: 42-48 [PMID: 15770587 DOI: 10.1053/j.sempedsurg.2004.10.024]

4   **Gallo A**, Esquivel CO. Current options for management of biliary atresia. *Pediatr Transplant* 2013; **17**: 95-98 [PMID: 23347466 DOI: 10.1111/petr.12040]

5   **Bessho K**, Bezerra JA. Biliary atresia: will blocking inflammation tame the disease? *Annu Rev Med* 2011; **62**: 171-185 [PMID: 21226614 DOI: 10.1146/annurev-med-042909-093734]

6   **Boulter L**, Lu WY, Forbes SJ. Differentiation of progenitors in the liver: a matter of local choice. *J Clin Invest* 2013; **123**: 1867-1873 [PMID: 23635784 DOI: 10.1172/JCI66026]

7   **Morell CM**, Strazzabosco M. Notch signaling and new therapeutic options in liver disease. *J Hepatol* 2014; **60**: 885-890 [PMID: 24308992 DOI: 10.1016/j.jhep.2013.11.028]

8   **Li L**, Krantz ID, Deng Y, Genin A, Banta AB, Collins CC, Qi M, Trask BJ, Kuo WL, Cochran J, Costa T, Pierpont ME, Rand EB, Piccoli DA, Hood L, Spinner NB. Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nat Genet* 1997; **16**: 243-251 [PMID: 9207788 DOI: 10.1038/ng0797-243]

9   **Oda T**, Elkahloun AG, Pike BL, Okajima K, Krantz ID, Genin A, Piccoli DA, Meltzer PS, Spinner NB, Collins FS, Chandrasekharappa SC. Mutations in the human Jagged1 gene are responsible for Alagille syndrome. *Nat Genet* 1997; **16**: 235-242 [PMID: 9207787 DOI: 10.1038/ng0797-235]

10   **McCright B**, Lozier J, Gridley T. A mouse model of Alagille syndrome: Notch2 as a genetic modifier of Jag1 haploinsufficiency. *Development* 2002; **129**: 1075-1082 [PMID: 11861489]

11   **Kodama Y**, Hijikata M, Kageyama R, Shimotohno K, Chiba T. The role of notch signaling in the development of intrahepatic bile ducts. *Gastroenterology* 2004; **127**: 1775-1786 [PMID: 15578515 DOI: 10.1053/j.gastro.2004.09.004]

12   **Sumazaki R**, Shiojiri N, Isoyama S, Masu M, Keino-Masu K, Osawa M, Nakauchi H, Kageyama R, Matsui A. Conversion of biliary system to pancreatic tissue in Hes1-deficient mice. *Nat Genet* 2004; **36**: 83-87 [PMID: 14702043 DOI: 10.1038/ng1273]

13   **Zhang RZ**, Yu JK, Peng J, Wang FH, Liu HY, Lui VC, Nicholls JM, Tam PK, Lamb JR, Chen Y, Xia HM. Role of CD56-expressing immature biliary epithelial cells in biliary atresia. *World J Gastroenterol* 2016; **22**: 2545-2557 [PMID: 26937142 DOI: 10.3748/wjg.v22.i8.2545]

14   **de Boer CJ**, van Krieken JH, Janssen-van Rhijn CM, Litvinov SV. Expression of Ep-CAM in normal, regenerating, metaplastic, and neoplastic liver. *J Pathol* 1999; **188**: 201-206 [PMID: 10398165 DOI: 10.1002/(SICI)1096-9896(199906)188:2<201::AID-PATH339>3.0.CO;2-8]

15   **Joplin R,** Kachilele S. Human intrahepatic biliary epithelial cell lineages: studies in vitro. Hepatocyte Transplantation: Springer, 2009: 193-206

16   **Sekiya S**, Suzuki A. Intrahepatic cholangiocarcinoma can arise from Notch-mediated conversion of hepatocytes. *J Clin Invest* 2012; **122**: 3914-3918 [PMID: 23023701 DOI: 10.1172/JCI63065]

17   **Stamp L**, Crosby HA, Hawes SM, Strain AJ, Pera MF. A novel cell-surface marker found on human embryonic hepatoblasts and a subpopulation of hepatic biliary epithelial cells. *Stem Cells* 2005; **23**: 103-112 [PMID: 15625127 DOI: 10.1634/stemcells.2004-0147]

18   **Arnold M**, Patton JT, McDonald SM. Culturing, storage, and quantification of rotaviruses. *Curr Protoc Microbiol* 2009; **15**: Unit 15C.3 [PMID: 19885940 DOI: 10.1002/9780471729259.mc15c03s15]

19   **Dollé L**, Theise ND, Schmelzer E, Boulter L, Gires O, van Grunsven LA. EpCAM and the biology of hepatic stem/progenitor cells. *Am J Physiol Gastrointest Liver Physiol* 2015; **308**: G233-G250 [PMID: 25477371 DOI: 10.1152/ajpgi.00069.2014]

20   **Yoon SM**, Gerasimidou D, Kuwahara R, Hytiroglou P, Yoo JE, Park YN, Theise ND. Epithelial cell adhesion molecule (EpCAM) marks hepatocytes newly derived from stem/progenitor cells in humans. *Hepatology* 2011; **53**: 964-973 [PMID: 21319194 DOI: 10.1002/hep.24122]

21   **Zhang Q**, Zhang CS, Xin Q, Ma Z, Liu GQ, Liu BB, Wang FM, Gao YT, Du Z. Perinodular ductular reaction/epithelial cell adhesion molecule loss in small hepatic nodules. *World J Gastroenterol* 2014; **20**: 10908-10915 [PMID: 25152593 DOI: 10.3748/wjg.v20.i31.10908]

22   **Gao J**, Yan Q, Wang J, Liu S, Yang X. Epithelial-to-mesenchymal transition induced by TGF-β1 is mediated by AP1-dependent EpCAM expression in MCF-7 cells. *J Cell Physiol* 2015; **230**: 775-782 [PMID: 25205054 DOI: 10.1002/jcp.24802]

23   **Massoner P**, Thomm T, Mack B, Untergasser G, Martowicz A, Bobowski K, Klocker H, Gires O, Puhr M. EpCAM is overexpressed in local and metastatic prostate cancer, suppressed by chemotherapy and modulated by MET-associated miRNA-200c/205. *Br J Cancer* 2014; **111**: 955-964 [PMID: 24992580 DOI: 10.1038/bjc.2014.366]

24   **Kalluri R**, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest* 2003; **112**: 1776-1784 [PMID: 14679171 DOI: 10.1172/JCI20530]

25   **Williams MJ**, Clouston AD, Forbes SJ. Links between hepatic fibrosis, ductular reaction, and progenitor cell expansion. *Gastroenterology* 2014; **146**: 349-356 [PMID: 24315991 DOI: 10.1053/j.gastro.2013.11.034]

26   **Omenetti A**, Bass LM, Anders RA, Clemente MG, Francis H, Guy CD, McCall S, Choi SS, Alpini G, Schwarz KB, Diehl AM, Whitington PF. Hedgehog activity, epithelial-mesenchymal transitions, and biliary dysmorphogenesis in biliary atresia. *Hepatology* 2011; **53**: 1246-1258 [PMID: 21480329 DOI: 10.1002/hep.24156]

27   **Xiao Y**, Zhou Y, Chen Y, Zhou K, Wen J, Wang Y, Wang J, Cai W. The expression of epithelial-mesenchymal transition-related proteins in biliary epithelial cells is associated with liver fibrosis in biliary atresia. *Pediatr Res* 2015; **77**: 310-315 [PMID: 25406900 DOI: 10.1038/pr.2014.181]

28   **Deng YH**, Pu CL, Li YC, Zhu J, Xiang C, Zhang MM, Guo CB. Analysis of biliary epithelial-mesenchymal transition in portal tract fibrogenesis in biliary atresia. *Dig Dis Sci* 2011; **56**: 731-740 [PMID: 20725787 DOI: 10.1007/s10620-010-1347-6]

29   **Roncarati R**, Sestan N, Scheinfeld MH, Berechid BE, Lopez PA, Meucci O, McGlade JC, Rakic P, D'Adamio L. The gamma-secretase-generated intracellular domain of beta-amyloid precursor protein binds Numb and inhibits Notch signaling. *Proc Natl Acad Sci U S A* 2002; **99**: 7102-7107 [PMID: 12011466 DOI: 10.1073/pnas.102192599]

30   **Fiorotto R**, Raizner A, Morell CM, Torsello B, Scirpo R, Fabris L, Spirli C, Strazzabosco M. Notch signaling regulates tubular morphogenesis during repair from biliary damage in mice. *J Hepatol* 2013; **59**: 124-130 [PMID: 23500150 DOI: 10.1016/j.jhep.2013.02.025]

31   **Kohn A**, Dong Y, Mirando AJ, Jesse AM, Honjo T, Zuscik MJ, O'Keefe RJ, Hilton MJ. Cartilage-specific RBPjκ-dependent and -independent Notch signals regulate cartilage and bone development. *Development* 2012; **139**: 1198-1212 [PMID: 22354840 DOI: 10.1242/dev.070649]

32   **Zhang L**, Zhu C, Guo Y, Wei F, Lu J, Qin J, Banerjee S, Wang J, Shang H, Verma SC, Yuan Z, Robertson ES, Cai Q. Inhibition of KAP1 enhances hypoxia-induced Kaposi's sarcoma-associated herpesvirus reactivation through RBP-Jκ. *J Virol* 2014; **88**:

6873-6884 [PMID: 24696491 DOI: 10.1128/JVI.00283-14]

33   **Hsieh JJ**, Nofziger DE, Weinmaster G, Hayward SD. Epstein-Barr virus immortalization: Notch2 interacts with CBF1 and blocks differentiation. *J Virol* 1997; **71**: 1938-1945 [PMID: 9032325]

34   **Mills CO**, Freeman JF, Salt PJ, Elias E. Effect of anaesthetic agents on bile flow and biliary excretion of 131I-cholylglycyltyrosine in the rat. *Br J Anaesth* 1989; **62**: 311-315 [PMID: 2784686 DOI: 10.1093/bja/62.3.311]

High expression of type I inositol 1,4,5-trisphosphate receptor in the kidney of rats with hepatorenal syndrome

Jing-Bo Wang, Ye Gu, Ming-Xiang Zhang, Shun Yang, Yan Wang, Wei Wang, Xi-Ran Li, Yi-Tong Zhao, Hai-Tao Wang

Jing-Bo Wang, Ye Gu, Ming-Xiang Zhang, Yan Wang, Wei Wang, Xi-Ran Li, Yi-Tong Zhao, Liver Cirrhosis Ward, the Sixth People's Hospital of Shenyang, Shenyang 110006, Liaoning Province, China

Shun Yang, Liaoning Cancer Hospital & Institute, Shenyang 110042, Liaoning Province, China

Hai-Tao Wang, Department of General Surgery, the Second Affiliated Hospital of Shenyang Medical College, Shenyang 110002, Liaoning Province, China

ORCID number: Jing-Bo Wang (0000-0001-9207-6245); Ye Gu (0000-0002-3798-1119); Ming-Xiang Zhang (0000-0001-6519-3497); Shun Yang (0000-0003-4743-0588); Yan Wang (0000-0002-0427-3458); Wei Wang (0000-0002-4964-3280); Xi-Ran Li (0000-0002-3253-407X); Yi-Tong Zhao (0000-0002-5059-8608); Hai-Tao Wang (0000-0002-7385-0642).

Abstract

AIM

To detect the expression of type I inositol 1,4,5-trisphosphate receptor (IP_3RI) in the kidney of rats with hepatorenal syndrome (HRS).

METHODS

One hundred and twenty-five Sprague-Dawley rats were randomly divided into four groups to receive an intravenous injection of D-galactosamine (D-GalN) plus lipopolysaccharide (LPS; group G/L, $n = 50$), D-GalN alone (group G, $n = 25$), LPS alone (group L, $n = 25$), and normal saline (group NS, $n = 25$), respectively.

At 3, 6, 9, 12, and 24 h after injection, blood, liver, and kidney samples were collected. Hematoxylin-eosin staining of liver tissue was performed to assess hepatocyte necrosis. Electron microscopy was used to observe ultrastructural changes in the kidney. Western blot analysis and real-time PCR were performed to detect the expression of IP₃RI protein and mRNA in the kidney, respectively.

RESULTS
Hepatocyte necrosis was aggravated gradually, which was most significant at 12 h after treatment with D-galactosamine/lipopolysaccharide, and was characterized by massive hepatocyte necrosis. At the same time, serum levels of biochemical indicators including liver and kidney function indexes were all significantly changed. The structure of the renal glomerulus and tubules was normal at all time points. Western blot analysis indicated that IP₃RI protein expression began to rise at 3 h ($P < 0.05$) and peaked at 12 h ($P < 0.01$). Real-time PCR demonstrated that IP₃RI mRNA expression began to rise at 3 h ($P < 0.05$) and peaked at 9 h ($P < 0.01$).

CONCLUSION
IP₃RI protein expression is increased in the kidney of HRS rats, and may be regulated at the transcriptional level.

Key words: Hepatorenal syndrome; Type I inositol 1,4,5-trisphosphate receptor; Glomerular mesangial cells; Vascular smooth muscle cells

Core tip: Type I inositol 1,4,5-trisphosphate receptor (IP₃RI) protein expression is increased in the kidney of hepatorenal syndrome (HRS) rats, and IP₃RI protein expression may be regulated at the transcriptional level. Increased expression of IP₃RI may be closely associated with HRS development and progression through excessive renal vascular contraction resulting in insufficient renal blood perfusion.

INTRODUCTION
Hepatorenal syndrome (HRS), one of the most severe complications of liver failure (LF) and the leading cause of death in LF[1], is functional renal failure secondary to LF[2-10]. At present, the exact pathogenesis of HRS is still unclear, and the reduction in renal blood flow induced by renal vasoconstriction is considered to play a central role in the development of HRS[11,12]. Renal blood flow is regulated by the contraction and relaxation of vascular smooth muscle cells (VSMCs) of glomerular afferent arteries and glomerular mesangial cells (GMCs), while the contraction and relaxation of VSMCs and GMCs are regulated by intracellular Ca^{2+} concentrations[13,14]. GMCs are in direct contact with glomerular endothelial cells. When GMCs contract, glomerular mesangial volume decreases by 20%-25%, glomerular capillary plexuses are reduced, and the area for glomerular filtration is reduced. Inositol 1,4,5-trisphosphate (IP₃) receptor (IP₃Rs) is the main Ca^{2+} release channel in cells. IP₃ is an intercellular second messenger mediating transmembrane signal transmission. When binding to IP₃Rs, IP₃ mediates intracellular calcium release and extracellular calcium influx[15,16]. VSMCs and GMCs transmit extracellular signals into the cell *via* the IP₃-IP₃R pathway, increasing intracellular Ca^{2+} concentrations[17,18]. Is high expression of renal IP₃Rs associated with HRS? To answer this question, in the present study we detected the expression of IP₃RI protein and mRNA in the kidney of HRS rats to determine the relationship between IP₃RI expression and HRS.

MATERIALS AND METHODS

Materials
Specific pathogen-free (SPF) Sprague-Dawley (SD) rats, weighing 220 g ± 20 g, were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences (Animal Certificate No. SCXK-2017-004; Beijing, China). Prior to experimentation, the rats were reared in separate cages at 23 ℃ ± 3 ℃ under a 12 h/12 h light/dark cycle, with free access to ordinary chow (purchased from the Laboratory Animal Center of China Medical University, Shenyang, China) and water. After one week of adaptation, the rats were used in the experiments.

D-galactosamine (D-GalN) and lipopolysaccharide (LPS) were purchased from Sigma (St. Louis, MO, United States). Anti-IP₃RI antibody was obtained from US Biological (St. Salem, OR, United States). An enhanced chemiluminescence (ECL) kit was purchased from Pierce, Dallas, TX, United States. RNAiso™ plus, Prime Script TM ᴿᵀ Reagent Kit, and SYBR® Premix EX Tag™ were purchased from TakaRa (Shiga, Japan).

Rat model of HRS
One hundred and twenty-five SD rats of SPF grade, weighing 220 ± 20 g, were randomly divided into four groups to receive an intravenous injection of D-GalN plus LPS (group G/L), D-GalN alone (group G), LPS alone (group L), and normal saline (group NS), respectively. Each group was further divided into five subgroups for testing at different time points (3, 6, 9,

12, and 24 h). Group G/L contained ten rats at each time point, and the other groups contained five rats at each time point. The rats were weighed and then injected with D-GalN (400 mg/kg body weight) and/or LPS (32 μg/kg) or NS (2 mL/kg) *via* the tail vein. Rats that died during the modeling process were excluded from the study. At 3, 6, 9, 12, and 24 h after modeling, the rats in groups G/L, G, and L were anesthetized with 0.8% pentobarbital sodium at 40 mg/kg *via* intraperitoneal injection and sacrificed to obtain liver and kidney tissues. A section of each tissue was fixed in formalin, and the remainder was preserved at -80 ℃ for Western blot and real-time PCR analysis of IP₃RI protein and mRNA expression, respectively.

Western blot analysis
For total protein preparation, renal tissue was lysed for 15 min in a lysis solution containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA, 5 mg/mL leupeptin, sodium orthovanadate, sodium fluoride, and 1 mmol/L PMSF, and then centrifuged at 12000 rpm for 12 min. The supernatant was collected and preserved at -80 ℃.

After total protein concentration was determined using the bicinchoninic acid (BCA) method, the protein samples were mixed with 5 × loading buffer at a ratio of 4:1 (v/v), boiled for 5 min, resolved by 8% SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes. The membranes were then blocked with 5% skimmed milk, Tris-buffered saline and Tween-20, and incubated with primary antibody against IP₃RI (dilution, 1:1000) at 4 ℃ overnight. This was followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (dilution, 1:3000) for 2 h at room temperature. The immunoblots were visualized using an enhanced chemiluminescence system. The molecular weight of the target band was 230 kDa. β-actin (45 kDa) was used as an internal control. Digital imaging software was used for densitometry analyses, and the relative IP₃RI level was calculated as IP₃RI grey value divided by β-actin grey value.

Real-time PCR
Total RNA was prepared from renal tissue using Trizol according to the manufacturer's instructions. After reverse transcription to cDNA in a 10-μL system containing 2.0 μL of 5 × Prime Script™ Buffer (for real time), 0.5 μL of Prime Script™ RT Enzyme Mi, 0.5 μL of Oligo dT Primer (50 μmol/L), 0.5 μL of Random 6-mers (100 μmol/L), 5.5 μL of RNase Free dH₂O, and 1.0 μL of RNA (500 ng/μL), real-time PCR was performed in a 25-μL system containing 12.5 μL of 2 × SYBR Premix Ex Tag™, 0.5 μL of PCR Forward Primer (10 μmol/L), 0.5 μL of PCR Reverse Primer (10 μmol/L), 9.5 μL of RNase Free dH₂O, and 2.0 μL of cDNA. Cycling parameters were 95 ℃ for 30 s and 45 cycles of 95 ℃ for 5 s, 57 ℃

for 20 s, and 72 ℃ for 30 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference. The primers used were as follows: forward, 5′-TCTGGCCAGCTGTCAGAACTAAAG-3′ and reverse, 5′-GTGGGTTGACATTCATGTGAGGA-3′ for IP₃RI, and forward, 5′-GACAACTTTGGCATCGTGGA-3′ and reverse, 5′-GACAACTTTGGCATCGTGGA-3′ for GAPDH. The double-standard curve method was used to determine the relative IP₃RI mRNA expression.

Statistical analysis
All statistical analyses were performed using SPSS 13.0 software. Numerical data, expressed as mean ± standard error of the mean, were compared using analysis of variance. *P* values < 0.05 were considered statistically significant.

RESULTS

Successful induction of HRS in rats with D-GalN/LPS
Following intravenous injection of D-GalN at 400 mg/kg body weight combined with LPS at 32 μg/kg in male SD rats, HRS was successfully induced. Twelve hours after injection, glomerular filtration rate (GFR) significantly decreased, liver and kidney function were severely impaired, and serum biochemical indices, such as alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine (Cr), exhibited significant changes. Hematoxylin-eosin staining showed massive hepatocyte necrosis with severe hemorrhage (Figure 1), while renal tissue had a normal morphology at the various time points (Figure 2). These changes were consistent with the clinical features of HRS.

Western blot analysis of IP₃RI protein expression
IP₃RI (230 kDa) and β-actin (45 kDa) were detected in all groups. Densitometry analyses showed that IP₃RI protein expression was significantly elevated in group G/L compared with group NS. This elevation began at 3 h (1.46 ± 0.07 *vs* 1.00 ± 0.05, *P* = 0.011), became obvious at 9 h, and reached a peak at 12 h (2.89 ± 0.14 *vs* 1.00 ± 0.05, *P* = 0.000) (Figure 3A).

At 12 h, the expression of IP₃RI protein in the kidney was significantly higher in group G/L than in groups G (1.17 ± 0.08) and L (1.02 ± 0.09) (*P* = 0.000 for both), thus excluding the impact of D-GalN or LPS on the expression of IP₃RI protein. There was no significant difference in IP₃RI protein expression between groups G and L (*P* = 0.245) or between group G or L and group NS (*P* > 0.05 for both) (Figure 3B).

RT-PCR analysis of IP₃RI mRNA expression
IP₃RI mRNA expression was significantly elevated in group G/L compared with group NS. This elevation began at 3 h (2.89 ± 0.51 *vs* 1.00 ± 0.00, *P* = 0.05), became obvious at 6 h (5.01 ± 0.38, *P* = 0.000), and reached a peak at 9 h (9.96 ± 0.63, *P* = 0.000). IP₃RI mRNA expression began to decline at 12 h, and at 24

Figure 1 Histopathology of the liver (HE staining, × 200). A: Group Normal Saline (NS). Normal hepatocytes were arranged in cords; B: Group D-galactosamine (D-GalN) plus lipopolysaccharide (LPS) (G/L). At 12 h, massive hepatocyte necrosis with severe hemorrhage developed; C: Group D-GalN (G). At 12 h, spotty hepatocyte necrosis was observed; C: Group LPS (L). At 12 h, hepatocytes began to develop necrosis, with incomplete necrosis visible.

Figure 2 Histopathology of the kidney. A: The glomerular basement membrane of the kidney was intact, and the foot processes of podocytes and fenestra of endothelial cells were clearly visible; B: The basal part of proximal tubule cubical epithelial cells had abundant plasma membrane infolding, which was rich in longitudinally arranged mitochondria with intact cristae. On the free surface of proximal tubule cubical epithelial cells, microvilli were long and dense; C: The basal part of distal tubule cubical epithelial cells also had abundant plasma membrane infolding, which was rich in mitochondria. On the free surface of distal tubule cubical epithelial cells, microvilli were short and sparse.

h, it returned to the level observed at 6 h. IP$_3$RI mRNA expression at 9 h was significantly higher in group G/L than in groups G (1.43 ± 0.18) and L (1.29 ± 0.17) (P = 0.000 for both; Figure 4). IP$_3$RI mRNA expression did not differ significantly between group G or L and group NS (P > 0.05 for both).

DISCUSSION

HRS is one of the most common and severe complications of fulminant liver failure (FHF) and an advanced liver disease, with approximately 55% of FHF patients developing HRS[19,20]. The pathogenesis of HRS is still not completely clear, although it is believed to be associated with excessive renal vascular contraction, insufficient renal blood perfusion, sympathetic nervous system activation, and increased synthesis of vasoactive substances, all of which make the kidneys more sensitive to low perfusion[21,22]. Renal blood flow and GFR decrease significantly in HRS due to renal vasoconstriction, and many factors are involved in this process. A significant increase in vasocontracting factors

Figure 3 Expression of type Ⅰ inositol 1,4,5-trisphosphate receptor (IP₃RI) protein in the kidney of rats in each group. A: The expression of IP₃RI protein in the kidney significantly increased in group D-galactosamine (D-GalN) plus lipopolysaccharide (LPS) (G/L), and was especially prominent at 12 h [$^aP < 0.05$ *vs* group Normal Saline (NS)]. B: The expression of IP₃RI protein in the kidney significantly increased in group G/L compared with the other groups ($^aP < 0.05$ *vs* group NS, $^cP < 0.05$ *vs* group G/L).

[*e.g.*, endothelin (ET) and angiotensin II] in the blood not only leads to renal vascular contraction, but also decreases the glomerular filtration coefficient (K_f) and GFR[23,24]. The contraction of VSMCs results in reduced renal blood flow, while GMC contraction reduces the glomerular filtration fraction and coefficient. As both VSMCs and GMCs are extremely sensitive to vasoactive substances, GFR is significantly decreased in HRS. ET and angiotensin II are important renal vasocontracting factors, and they activate Ca^{2+} channels *via* the IP₃-IP₃Rs pathway. IP₃Rs is the intracellular calcium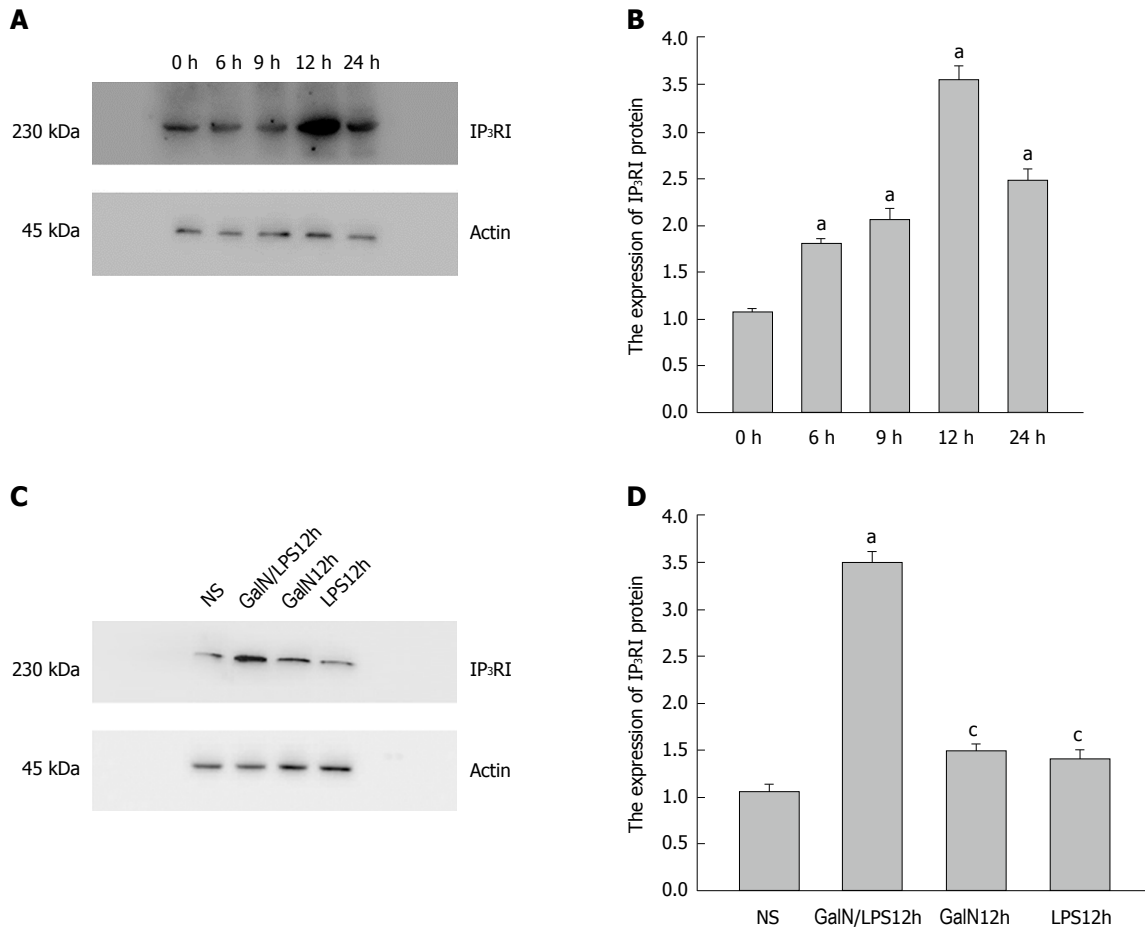 reservoir, which is present mainly in the endoplasmic reticulum and on the membrane, directly or indirectly mediating the calcium influx[25-27]. In addition, IP₃Rs is also present in the nucleus, participating in nuclear calcium release and regulating gene expression[28,29]. When IP₃ binds to IP₃Rs, a conformational change in IP₃Rs occurs, the calcium channel is open, and the calcium reserve in the endoplasmic reticulum is released into the cytoplasm. As a result, cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) increases, thus causing cell contraction[30-32]. Therefore, IP₃Rs mediates an important Ca^{2+} signaling pathway in the cell, and the expression of IP₃Rs is closely related

to the sensitivity of the kidney to vasoconstrictors[33-35]. IP₃Rs has four types of ligand binding sites associated with calcium channels[36-39], and renal IP₃RI is mainly found in GMCs and VSMCs, and there is almost no IP₃RI on the surface of other renal cells[40,41]. Therefore, the expression levels of IP₃RI in renal GMCs and VSMCs may be related to renal vasoconstriction. As the opening of IP₃-IP₃Rs channels can increase intracellular $[Ca^{2+}]_i$, theoretically the expression level of IP₃RI is closely related to the intracellular $[Ca^{2+}]_i$ level. Wang *et al* observed increased expression of IP₃RI in the glomerular capillary loops and anterior artery of rats with liver cirrhosis by immunohistochemistry. However, it is unknown whether the expression of IP₃RI increases in FHF. To answer this question, we detected IP₃RI expression in the renal tissue of a rat model of FHF at different time points at both the protein and mRNA levels using Western blot and real-time quantitative PCR, respectively.

Semi-quantitative Western blot analysis demonstrated that IP₃RI protein expression was low in normal kidney tissue. Following treatment with D-GalN plus LPS, IP₃RI protein expression began to rise at 3 h and
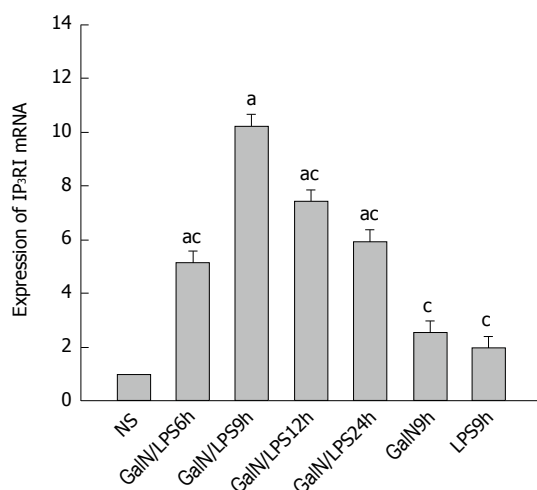
Figure 4 Expression of type 1 inositol 1,4,5-trisphosphate receptor (IP₃RI) mRNA in the kidney of rats in group D-galactosamine (D-GalN) plus lipopolysaccharide (LPS) (G/L). The expression of IP₃RI mRNA in the kidney significantly increased in group G/L, and was especially prominent at 12 h ($^aP < 0.05$ *vs* group Normal Saline (NS), $^cP < 0.05$ *vs* group G/L).

reached a peak at 12 h. Interestingly, liver and kidney dysfunction and hepatocyte necrosis were most severe and blood TNF-α and ET-1 levels were highest at 12 h, which were concomitant with the elevation of IP₃RI protein expression in the kidney[42,43]. By treating the animals with D-GalN or LPS alone, we excluded the effect of these drugs on IP₃RI protein expression. As HRS developed 12 h after D-GalN/LPS administration, we compared the IP₃RI protein expression at this time point among the groups. The results showed that IP₃RI protein expression was high in group G/L and low in groups G, L, and NS.

In order to understand whether IP₃RI protein expression in the kidney is regulated at the transcriptional level, real-time quantitative PCR was performed. The fluorescent dye SYBR Green I[44,45] added to the PCR reaction system can be incorporated into double-stranded DNA with PCR amplification and markedly enhance fluorescence[46-48]. The relative expression levels of these two parameters were calculated by the housekeeping gene GAPDH. It was found that the relative expression of IP₃RI mRNA to GAPDH mRNA began to rise 3 h after D-GalN and LPS administration, but the protein level did not rise at this time point. At 9 h, the expression level of IP₃RI mRNA reached the highest level. Although the protein level was also high at this time point, it was lower than that at 12 h. The expression of IP₃RI mRNA began to decrease, but it was still significantly higher than that in the control group. These changes can be explained from two aspects. On the one hand, IP₃RI mRNA expression may be prior to protein expression, which is associated not only with the translation efficiency and the speed of mRNA degradation, but also with the rate of protein degradation. On the other hand, the protein synthesis process also includes the assembly and translocation of proteins in ribosomes, which may affect the final expression of IP₃RI protein.

In conclusion, joint D-GalN/LPS administration can induce HRS in SD rats at 12 h, which is concomitant with peak IP₃RI protein expression in the kidney. Increased IP₃RI protein expression may be regulated at the transcriptional level. Thus, increased expression of IP₃RI may be closely associated with HRS development and progression.

ARTICLE HIGHLIGHTS

Research background

Hepatorenal syndrome (HRS) is one of the common and severe complications of liver failure and advanced liver disease, with approximately 55% of these patients developing this severe complication. At present, HRS has unclear pathogenesis, limited treatment options, and poor therapeutic efficacy. Once renal dysfunction aggravates rapidly, 60%-80% of patients with HRS will die. Therefore, elucidating the mechanism underlying the development and progression of HRS and taking effective preventive and therapeutic measures may improve the success rate of rescue, the incidence rate, and the mortality rate of HRS.

Research motivation

To detect the protein and mRNA expression of type 1 inositol 1,4,5-trisphosphate receptor (IP₃RI) in the kidney of rats with HRS by Western blot and real-time PCR.

Research objectives

To explore whether high expression of renal IP₃RI is associated with Ca^{2+} influx in vascular smooth muscle cells of glomerular afferent arteries and glomerular mesangial cells in rats with HRS.

Research methods

D-galactosamine (D-GalN) and/or lipopolysaccharide (LPS) were used to treat male Sprague-Dawley (SD) rats *via* the tail vein. Twelve hours after injection, massive hepatocyte necrosis with severe hemorrhage occurred in the liver, while renal tissue had a normal morphology. In addition, liver and kidney function was impaired severely, and serum biochemical indexes exhibited significant changes. These changes were consistent with the clinical features of HRS. Western blot and real-time PCR were then used to detect the protein and mRNA expression of renal IP₃RI, respectively.

Research results

IP₃RI protein expression was significantly elevated in rats with HRS. The elevation began at 3 h and reached the peak at 12 h. IP₃RI mRNA expression was also significantly elevated in rats with HRS. The elevation began at 3 h and peaked at 9 h.

Research conclusions

Joint D-GalN/LPS administration can induce HRS in SD rats at 12 h, which is concomitant with peaked IP₃RI protein and mRNA expression in the kidney. Increased expression of IP₃RI may be closely associated with HRS development and progression.

Research perspectives

Our results suggest that IP₃RI may be a signal molecule involved in the reduction of renal blood flow induced by renal vasoconstriction in HRS, thus providing a theoretical basis for further research of the pathogenesis of HRS. Gene silencing technology may be adopted to further elucidate the role of IP₃RI in the pathogenesis of HRS.

REFERENCES

1   **Hernaez R**, Solà E, Moreau R, Ginès P. Acute-on-chronic liver failure: an update. *Gut* 2017; **66**: 541-553 [PMID: 28053053 DOI:

10.1136/gutjnl-2016-312670]

2   **de Mattos ÁZ**, de Mattos AA, Méndez-Sánchez N. Hepatorenal syndrome: Current concepts related to diagnosis and management. *Ann Hepatol* 2016; **15**: 474-481 [PMID: 27236146]

3   **Bittencourt PL**, Farias AQ, Terra C. Renal failure in cirrhosis: Emerging concepts. *World J Hepatol* 2015; **7**: 2336-2343 [PMID: 26413223 DOI: 10.4254/wjh.v7.i21.2336]

4   **Khan MQ**, Anand V, Hessefort N, Hassan A, Ahsan A, Sonnenberg A, Fimmel CJ. Utility of Electronic Medical record-based Fibrosis Scores in Predicting Advanced Cirrhosis in Patients with Hepatitic C Virus Infection. *J Transl Int Med* 2017; **5**: 43-48 [PMID: 28680838 DOI: 10.1515/jtim-2017-0011]

5   **Torres-Valadez R**, Roman S, Jose-Abrego A, Sepulveda-Villegas M, Ojeda-Granados C, Rivera-Iñiguez I, Panduro A. Early Detection of Liver Damage in Mexican Patients with Chronic Liver Disease. *J Transl Int Med* 2017; **5**: 49-57 [PMID: 28680839 DOI: 10.1515/jtim-2017-0003]

6   **Abenavoli L**, Milic N, Luzza F, Boccuto L, De Lorenzo A. Polyphenols Treatment in Patients with Nonalcoholic Fatty Liver Disease. *J Transl Int Med* 2017; **5**: 144-147 [PMID: 29164049 DOI: 10.1515/jtim-2017-0027]

7   **Elbahrawy A**, Elwassief A, Abdallah AM, Kasem A, Mostafa S, Makboul K, Ali MS, Alashker A, Eliwa AM, Shahbah H, Othman MA, Morsy MH, Abdelbaseer MA, Abdelhafeez H. Hepatitis C Virus Exposure Rate among Health-care Workers in Rural Lower Egypt Governorates. *J Transl Int Med* 2017; **5**: 164-168 [PMID: 29085789 DOI: 10.1515/jtim-2017-0024]

8   **Das UN**. Renin-angiotensin-aldosterone system in insulin resistance and metabolic syndrome. *J Transl Int Med* 2016; **4**: 66-72 [PMID: 28191524 DOI: 10.1515/jtim-2016-0022]

9   **Bao H**, Peng A. The Green Tea Polyphenol(-)-epigallocatechin-3-gallate and its beneficial roles in chronic kidney disease. *J Transl Int Med* 2016; **4**: 99-103 [PMID: 28191529 DOI: 10.1515/jtim-2016-0031]

10   **Wang L**, Mohan C. Contrast-enhanced ultrasound: A promising method for renal microvascular perfusion evaluation. *J Transl Int Med* 2016; **4**: 104-108 [PMID: 28191530 DOI: 10.1515/jtim-2016-0033]

11   **Dundar HZ**, Yılmazlar T. Management of hepatorenal syndrome. *World J Nephrol* 2015; **4**: 277-286 [PMID: 25949942 DOI: 10.5527/wjn.v4.i2.277]

12   **Colle I**, Laterre PF. Hepatorenal syndrome: the clinical impact of vasoactive therapy. *Expert Rev Gastroenterol Hepatol* 2018; **12**: 173-188 [PMID: 29258378 DOI: 10.1080/17474124.2018.1417034]

13   **Geyer M**, Huang F, Sun Y, Vogel SM, Malik AB, Taylor CW, Komarova YA. Microtubule-Associated Protein EB3 Regulates IP3 Receptor Clustering and Ca(2+) Signaling in Endothelial Cells. *Cell Rep* 2015; **12**: 79-89 [PMID: 26119739 DOI: 10.1016/j.celrep.2015.06.001]

14   **Taylor CW**, Tovey SC, Rossi AM, Lopez Sanjurjo CI, Prole DL, Rahman T. Structural organization of signalling to and from IP3 receptors. *Biochem Soc Trans* 2014; **42**: 63-70 [PMID: 24450629 DOI: 10.1042/BST20130205]

15   **Chandrasekhar R**, Alzayady KJ, Yule DI. Using concatenated subunits to investigate the functional consequences of heterotetrameric inositol 1,4,5-trisphosphate receptors. *Biochem Soc Trans* 2015; **43**: 364-370 [PMID: 26009177 DOI: 10.1042/BST20140287]

16   **Alzayady KJ**, Wang L, Chandrasekhar R, Wagner LE 2nd, Van Petegem F, Yule DI. Defining the stoichiometry of inositol 1,4,5-trisphosphate binding required to initiate Ca2+ release. *Sci Signal* 2016; **9**: ra35 [PMID: 27048566 DOI: 10.1126/scisignal.aad6281]

17   **Ambudkar IS**. Ca^{2+} signaling and regulation of fluid secretion in salivary gland acinar cells. *Cell Calcium* 2014; **55**: 297-305 [PMID: 24646566 DOI: 10.1016/j.ceca.2014.02.009]

18   **Provence A**, Rovner ES, Petkov GV. Regulation of transient receptor potential melastatin 4 channel by sarcoplasmic reticulum inositol trisphosphate receptors: Role in human detrusor smooth muscle function. *Channels* (Austin) 2017; **11**: 459-466 [PMID: 28644055 DOI: 10.1080/19336950.2017.1341023]

19   **Bajaj JS**, O'Leary JG, Reddy KR, Wong F, Biggins SW, Patton H, Fallon MB, Garcia-Tsao G, Maliakkal B, Malik R, Subramanian RM, Thacker LR, Kamath PS; North American Consortium For The Study Of End-Stage Liver Disease (NACSELD). Survival in infection-related acute-on-chronic liver failure is defined by extrahepatic organ failures. *Hepatology* 2014; **60**: 250-256 [PMID: 24677131 DOI: 10.1002/hep.27077]

20   **Lenz K**, Buder R, Kapun L, Voglmayr M. Treatment and management of ascites and hepatorenal syndrome: an update. *Therap Adv Gastroenterol* 2015; **8**: 83-100 [PMID: 25729433 DOI: 10.1177/1756283X14564673]

21   **Piano S**, Schmidt HH, Ariza X, Amoros A, Romano A, Hüsing-Kabar A, Solà E, Gerbes A, Bernardi M, Alessandria C, Scheiner B, Tonon M, Maschmeier M, Solè C, Trebicka J, Gustot T, Nevens F, Arroyo V, Gines P, Angeli P; EASL CLIF Consortium, European Foundation for the Study of Chronic Liver Failure (EF Clif). Association Between Grade of Acute on Chronic Liver Failure and Response to Terlipressin and Albumin in Patients With Hepatorenal Syndrome. *Clin Gastroenterol Hepatol* 2018; Epub ahead of print [PMID: 29391267 DOI: 10.1016/j.cgh.2018.01.035]

22   **Tsien CD**, Rabie R, Wong F. Acute kidney injury in decompensated cirrhosis. *Gut* 2013; **62**: 131-137 [PMID: 22637695 DOI: 10.1136/gutjnl-2011-301255]

23   **Bekpinar S**, Vardagli D, Unlucerci Y, Can A, Uysal M, Gurdol F. Effect of rosiglitazone on asymmetric dimethylarginine metabolism in thioacetamide-induced acute liver injury. *Pathophysiology* 2015; **22**: 153-157 [PMID: 26224212 DOI: 10.1016/j.pathophys.2015.06.003]

24   **Sridharan K**, Sivaramakrishnan G. Vasoactive Agents for Hepatorenal Syndrome: A Mixed Treatment Comparison Network Meta-Analysis and Trial Sequential Analysis of Randomized Clinical Trials. *J Gen Intern Med* 2018; **33**: 97-102 [PMID: 28924736 DOI: 10.1007/s11606-017-4178-8]

25   **Ullah G**, Ullah A. Mode switching of Inositol 1,4,5-trisphosphate receptor channel shapes the Spatiotemporal scales of Ca2+ signals. *J Biol Phys* 2016; **42**: 507-524 [PMID: 27154029 DOI: 10.1007/s10867-016-9419-2]

26   **Alzayady KJ**, Sebé-Pedrós A, Chandrasekhar R, Wang L, Ruiz-Trillo I, Yule DI. Tracing the Evolutionary History of Inositol, 1, 4, 5-Trisphosphate Receptor: Insights from Analyses of Capsaspora owczarzaki Ca2+ Release Channel Orthologs. *Mol Biol Evol* 2015; **32**: 2236-2253 [PMID: 25911230 DOI: 10.1093/molbev/msv098]

27   **Bánsághi S**, Golenár T, Madesh M, Csordás G, RamachandraRao S, Sharma K, Yule DI, Joseph SK, Hajnóczky G. Isoform- and species-specific control of inositol 1,4,5-trisphosphate (IP3) receptors by reactive oxygen species. *J Biol Chem* 2014; **289**: 8170-8181 [PMID: 24469450 DOI: 10.1074/jbc.M113.504159]

28   **Okeke E**, Parker T, Dingsdale H, Concannon M, Awais M, Voronina S, Molgó J, Begg M, Metcalf D, Knight AE, Sutton R, Haynes L, Tepikin AV. Epithelial-mesenchymal transition, IP3 receptors and ER-PM junctions: translocation of Ca2+ signalling complexes and regulation of migration. *Biochem J* 2016; **473**: 757-767 [PMID: 26759379 DOI: 10.1042/BJ20150364]

29   **Subedi KP**, Son MJ, Chidipi B, Kim SW, Wang J, Kim KH, Woo SH, Kim JC. Signaling Pathway for Endothelin-1- and Phenylephrine-Induced cAMP Response Element Binding Protein Activation in Rat Ventricular Myocytes: Role of Inositol 1,4,5-Trisphosphate Receptors and CaMKII. *Cell Physiol Biochem* 2017; **41**: 399-412 [PMID: 28214885 DOI: 10.1159/000456422]

30   **Fan G**, Baker ML, Wang Z, Baker MR, Sinyagovskiy PA, Chiu W, Ludtke SJ, Serysheva II. Gating machinery of InsP3R channels revealed by electron cryomicroscopy. *Nature* 2015; **527**: 336-341 [PMID: 26458101 DOI: 10.1038/nature15249]

31   **Seo MD**, Velamakanni S, Ishiyama N, Stathopulos PB, Rossi AM, Khan SA, Dale P, Li C, Ames JB, Ikura M, Taylor CW. Structural and functional conservation of key domains in InsP3 and ryanodine receptors. *Nature* 2012; **483**: 108-112 [PMID: 22286060 DOI: 10.1038/nature10751]

32   **Alzayady KJ**, Wagner LE 2nd, Chandrasekhar R, Monteagudo A, Godiska R, Tall GG, Joseph SK, Yule DI. Functional inositol 1,4,5-trisphosphate receptors assembled from concatenated homo- and heteromeric subunits. *J Biol Chem* 2013; **288**: 29772-29784 [PMID: 23955339 DOI: 10.1074/jbc.M113.502203]

33   **Cao P**, Falcke M, Sneyd J. Mapping Interpuff Interval Distribution to the Properties of Inositol Trisphosphate Receptors. *Biophys J* 2017; **112**: 2138-2146 [PMID: 28538151 DOI: 10.1016/j.bpj.2017.03.019]

34   **Chandrasekhar R**, Alzayady KJ, Wagner LE 2nd, Yule DI. Unique Regulatory Properties of Heterotetrameric Inositol 1,4,5-Trisphosphate Receptors Revealed by Studying Concatenated Receptor Constructs. *J Biol Chem* 2016; **291**: 4846-4860 [PMID: 26755721 DOI: 10.1074/jbc.M115.705301]

35   **Yang J**, Vais H, Gu W, Foskett JK. Biphasic regulation of InsP3 receptor gating by dual Ca2+ release channel BH3-like domains mediates Bcl-xL control of cell viability. *Proc Natl Acad Sci USA* 2016; **113**: E1953-E1962 [PMID: 26976600 DOI: 10.1073/pnas.1517935113]

36   **Sun MY**, Geyer M, Komarova YA. IP3 receptor signaling and endothelial barrier function. *Cell Mol Life Sci* 2017; **74**: 4189-4207 [PMID: 28803370 DOI: 10.1007/s00018-017-2624-8]

37   **Golebiewska U**, Kay JG, Masters T, Grinstein S, Im W, Pastor RW, Scarlata S, McLaughlin S. Evidence for a fence that impedes the diffusion of phosphatidylinositol 4,5-bisphosphate out of the forming phagosomes of macrophages. *Mol Biol Cell* 2011; **22**: 3498-3507 [PMID: 21795401 DOI: 10.1091/mbc.E11-02-0114]

38   **Gulyás G**, Tóth JT, Tóth DJ, Kurucz I, Hunyady L, Balla T, Várnai P. Measurement of inositol 1,4,5-trisphosphate in living cells using an improved set of resonance energy transfer-based biosensors. *PLoS One* 2015; **10**: e0125601 [PMID: 25932648 DOI: 10.1371/journal.pone.0125601]

39   **Takeda Y**, Shimayoshi T, Holz GG, Noma A. Modeling analysis of inositol 1,4,5-trisphosphate receptor-mediated Ca2+ mobilization under the control of glucagon-like peptide-1 in mouse pancreatic β-cells. *Am J Physiol Cell Physiol* 2016; **310**: C337-C347 [PMID: 26741144 DOI: 10.1152/ajpcell.00234.2015]

40   **Bojjireddy N**, Botyanszki J, Hammond G, Creech D, Peterson R, Kemp DC, Snead M, Brown R, Morrison A, Wilson S, Harrison S, Moore C, Balla T. Pharmacological and genetic targeting of the PI4KA enzyme reveals its important role in maintaining plasma membrane phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate levels. *J Biol Chem*

2014; **289**: 6120-6132 [PMID: 24415756 DOI: 10.1074/jbc.M113.531426]

41   **Wagner LE 2nd**, Yule DI. Differential regulation of the InsP3 receptor type-1 and -2 single channel properties by InsP$_3$, Ca^{2+} and ATP. *J Physiol* 2012; **590**: 3245-3259 [PMID: 22547632 DOI: 10.1113/jphysiol.2012.228320]

42   **Wang JB**, Wang DL, Wang HT, Wang ZH, Wen Y, Sun CM, Zhao YT, Wu J, Liu P. Tumor necrosis factor-alpha-induced reduction of glomerular filtration rate in rats with fulminant hepatic failure. *Lab Invest* 2014; **94**: 740-751 [PMID: 24887412 DOI: 10.1038/labinvest.2014.71]

43   **Wang JB**, Wang HT, Li LP, Yan YC, Wang W, Liu JY, Zhao YT, Gao WS, Zhang MX. Development of a rat model of D-galactosamine/lipopolysaccharide induced hepatorenal syndrome. *World J Gastroenterol* 2015; **21**: 9927-9935 [PMID: 26379397 DOI: 10.3748/wjg.v21.i34.9927]

44   **Barbau-Piednoir E**, Bertrand S, Mahillon J, Roosens NH, Botteldoorn N. SYBR®Green qPCR Salmonella detection system allowing discrimination at the genus, species and subspecies levels. *Appl Microbiol Biotechnol* 2013; **97**: 9811-9824 [PMID: 24113820 DOI: 10.1007/s00253-013-5234-x]

45   **Barbau-Piednoir E**, Denayer S, Botteldoorn N, Dierick K, De Keersmaecker SCJ, Roosens NH. Detection and discrimination of five E. coli pathotypes using a combinatory SYBR® Green qPCR screening system. *Appl Microbiol Biotechnol* 2018; **102**: 3267-3285 [PMID: 29460001 DOI: 10.1007/s00253-018-8820-0]

46   **Barbau-Piednoir E**, Botteldoorn N, Mahillon J, Dierick K, Roosens NH. Fast and discriminative CoSYPS detection system of viable Salmonella spp. and Listeria spp. in carcass swab samples. *Int J Food Microbiol* 2015; **192**: 103-110 [PMID: 25440553 DOI: 10.1016/j.ijfoodmicro.2014.09.018]

47   **Acevedo AM**, Perera CL, Vega A, Ríos L, Coronado L, Relova D, Frías MT, Ganges L, Núñez JI, Pérez LJ. A duplex SYBR Green I-based real-time RT-PCR assay for the simultaneous detection and differentiation of Massachusetts and non-Massachusetts serotypes of infectious bronchitis virus. *Mol Cell Probes* 2013; **27**: 184-192 [PMID: 23810983 DOI: 10.1016/j.mcp.2013.06.001]

48   **Fellahi S**, El Harrak M, Kuhn JH, Sebbar G, Bouaiti el A, Khataby K, Fihri OF, El Houadfi M, Ennaji MM. Comparison of SYBR green I real-time RT-PCR with conventional agarose gel-based RT-PCR for the diagnosis of infectious bronchitis virus infection in chickens in Morocco. *BMC Res Notes* 2016; **9**: 231 [PMID: 27106608 DOI: 10.1186/s13104-016-2037-z]

ORIGINAL ARTICLE

Retrospective Study

Prognostic significance of the fibrinogen-to-albumin ratio in gallbladder cancer patients

Wei-Yu Xu, Hao-Hai Zhang, Jian-Ping Xiong, Xiao-Bo Yang, Yi Bai, Jian-Zhen Lin, Jun-Yu Long, Yong-Chang Zheng, Hai-Tao Zhao, Xin-Ting Sang

Wei-Yu Xu, Hao-Hai Zhang, Jian-Ping Xiong, Xiao-Bo Yang, Yi Bai, Jian-Zhen Lin, Jun-Yu Long, Yong-Chang Zheng, Hai-Tao Zhao, Xin-Ting Sang, Department of Liver Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China

ORCID number: Wei-Yu Xu (0000-0002-2101-4829); Hao-Hai Zhang (0000-0002-5292-6505); Jian-Ping Xiong (0000-0002-6163-2621); Xiao-Bo Yang (0000-0003-1929-8866); Yi Bai (0000-0002-1179-3734); Jian-Zhen Lin (0000-0002-4767-8834); Jun-Yu Long (0000-0001-5745-7165); Yong-Chang Zheng (0000-0002-6956-1186); Hai-Tao Zhao (0000-0002-3444-8044); Xin-Ting Sang (0000-0003-1952-0527).

Abstract

AIM
To investigate the prognostic role of fibrinogen-to-albumin ratio (FAR) on patients with gallbladder cancer (GBC) in this study.

METHODS
One hundred and fifty-four GBC patients were retro-

spectively analyzed, who received potentially curative cholecystectomy in our institute from March 2005 to December 2017. Receiver operating characteristic curve (ROC curve) was used to determine the optimal cut-offs for these biomarkers. In addition, Kaplan-Meier survival analysis as well as multivariate analysis were applied for prognostic analyses.

RESULTS
ROC curve revealed that the optimal cut-off value for FAR was 0.08. FAR was significantly correlated with age (P = 0.045), jaundice (P < 0.001), differentiation (P = 0.002), resection margin status (P < 0.001), T stage (P < 0.001), TNM stage (P < 0.001), and CA199 (P < 0.001) as well as albumin levels (P < 0.001). Multivariate analysis indicated that the resection margin status [hazard ratio (HR): 2.343, 95% confidence interval (CI): 1.532-3.581, P < 0.001], TNM stage (P = 0.035), albumin level (HR = 0.595, 95%CI: 0.385-0.921, P = 0.020) and FAR (HR: 2.813, 95%CI: 1.765-4.484, P < 0.001) were independent prognostic factors in GBC patients.

CONCLUSION
An elevated preoperative FAR was significantly correlated with unfavorable overall survival in GBC patients, while an elevated preoperative albumin level was a protective prognostic factor for patients with GBC. The preoperative FAR could be used to predict the prognosis of GBC patients, which was easily accessible, cost-effective and noninvasive.

Key words: Gallbladder cancer; Fibrinogen; Albumin; Fibrinogen-to-albumin ratio; Prognosis; Survival

Core tip: The vital prognostic significance of fibrinogen and serum albumin has been confirmed in diverse malignancies, presenting host hemostasis and nutrition, respectively. Moreover, elevated plasma fibrinogen and reduced serum albumin levels are significantly related to shortened survival of cancer patients. It is reported that fibrinogen-to-albumin ratio (FAR) is more potent in predicting cancer patient prognosis than elevated fibrinogen or reduced serum albumin level alone. Nevertheless, there has been no study on the prognostic role of FAR in gallbladder cancer (GBC). Herein, we defined an elevated preoperative FAR, featured by noninvasiveness, cost-effectiveness and easily-accessible, which was a potential prognostic indicator for GBC.

INTRODUCTION

Gallbladder cancer (GBC) is an uncommon malignancy among all types of cancer, but is the fifth most common gastrointestinal malignancy. Meanwhile, GBC is the most prevalent and aggressive cancer of the biliary tract[1-3]. Despite recent encouraging progress in the diagnosis and treatment of GBC, it is still a highly lethal disease, with overall 5-year survival rate under 5%[4]. Only surgical intervention renders probability of a long-term survival, however, most GBC patients generally present at late stage, with unresectable lesion. To be specific, fewer than 20% of cases are amenable to surgical treatment[5,6].

At present, a few clinicopathological parameters, such as clinical stage, performance status (PS), and pathological classification, have been demonstrated as independent survival predictors in patients harboring various types of common solid tumors[7]. Nevertheless, despite the wide application of high-resolution imaging systems, it is rather difficult to obtain accurate classification of clinical stage, and objective judgement of PS[8-10]. In addition, the pathological stage of tumor samples in these subjects is not as informative as that in untreated subjects[11]. In order to guarantee potent intense neoadjuvant therapy as well as regular follow-up in high-risk subjects, it is necessary to explore a simple and cost-effective predictor for the postoperative overall survival (OS) prior to surgery.

Accumulating evidence has demonstrated that nutritional deficiencies, hemostatic factors and systemic inflammatory response (SIR) are likely to be critically involved in the progression of human malignancies[12]. Fibrinogen plays an important regulatory role in both inflammation and cancer progression, including proliferation, angiogenesis as well as migration of tumor cells[13]. Serum albumin levels reflect the SIR of host and nutritional status[14-16]. Recently, accumulating researches have shown that both fibrinogen and serum albumin are important prognostic predictors in various cancers, and elevated plasma fibrinogen and lower serum albumin levels are significantly correlated with shorter survival in tumor patients[17-21].

From the results of the above studies, we can naturally hypothesize that the fibrinogen-to-albumin ratio (FAR) might be more powerful than elevated fibrinogen or lower serum albumin level in predicting the prognosis of patients with malignant tumors. In fact, Tan *et al*[22] have indicated that the preoperative FAR is an independent prognostic indicator for esophageal squamous-cell carcinoma (ESCC) patients, while Hwang *et al*[23] have indicated that the FAR is a more significant prognostic indicator than either indicator alone (elevated fibrinogen or lower serum albumin).

To our knowledge, there are no relevant studies concerning the prognostic significance of FAR in GBC patients. Herein, the study was designed to explore the prognostic roles of the preoperative FAR in GBC in terms of OS.

MATERIALS AND METHODS

Patients

Eligible patients were included in this study according to the following criteria: (1) patients with histological diagnosis of GBC; (2) GBC patients without other coexisting malignancies; (3) patients not undergoing other treatments before enrollment; (4) patients with complete clinical information and available follow-up data; and (5) patients aged > 18 years. The exclusion criteria were listed as follows: (1) patients with acute infection or chronic active inflammatory disease; (2) patients with collagen diseases, anemia and other diseases concerning the hematological system; (3) patients who received anticoagulant treatment or albumin transfusions before treatment; (4) patients with liver disease; and (5) patients with perioperative surgery-associated mortality. As a result, 154 GBC patients were retrospectively included and analyzed, who underwent potential curative resection at Peking Union Medical College Hospital of Chinese Academy of Medical Sciences and Peking Union Medical College (CAMS & PUMC) from January 2005 to May 2017.

Data collection

Baseline clinicopathological characteristics, including age, gender, comorbidities, ABO blood group, pathological classifications, tumor differentiation, resection margin status, maximal tumor diameter, TNM stage, and preoperative CA199, fibrinogen, and albumin levels. Patient age referred to the age at diagnosis of primary GBC. The eighth edition of the American Joint Committee on Cancer (AJCC-8th) TNM classification was utilized for TNM stage.

Ethical statement

The study was approved by the Medical Ethics Committee of Peking Union Medical College Hospital of CAMS & PUMC. All patients signed written informed consent. The study was carried out according to the ethical standard of the World Medical Association Declaration of Helsinki[24].

Fibrinogen and albumin measurements

Blood specimens were collected before breakfast within seven days before surgery, in order to assess the preoperative plasma fibrinogen and serum albumin concentrations. Afterwards, Datafai Fibrinogen (Sysmex Corporation, Kobe, Japan) and CA7000 analyzer (Sysmex Corporation, Kobe, Japan) were employed to assess fibrinogen level using the previously-mentioned Clauss method[25]. The normal reference values of plasma fibrinogen and serum albumin were 2-4 g/L and 35-51 g/L, respectively, according to relevant instructions.

FAR

FAR was defined by dividing the preoperative fibrinogen level by the preoperative serum albumin level.

Treatments and Follow up

All subjects received potential curative gallbladder resection at Peking Union Medical College Hospital of CAMS & PUMC. The extent of resection was classified as modified radical cholecystectomy or radical cholecystectomy and systemic therapy according to the extent of tumor invasion, which was identified by preoperative auxiliary examination results. Follow-up visits in our center were carried out every three months for the first two years, every six months for the third year and annually thereafter. The follow-up period was defined from the date of surgery to death or the last follow-up visit.

Statistical analysis

The continuous data with normal distribution were shown as the mean ± standard deviation (Kolmogorov-Smirnov test, $P > 0.05$), and those with abnormal distribution were expressed as the median (minimum-maximum). Frequencies and percentages were used for the categorical variables. Chi-square test or Fisher's exact test was utilized to assess differences in baseline clinicopathological characteristics between groups. OS referred to the duration from the date of surgery to death or the last follow-up visit. The optimal cut-off values of fibrinogen, albumin and FAR were identified by the receiver operating characteristic (ROC) curve. Kaplan-Meier method was used to generate the survival curves, followed by analysis by log-rank test. Additionally, multivariate Cox proportional hazards model was used to further assess those significant factors indicated by univariate analysis. SPSS version 24.0 (IBM Corp., Armonk, NY, United States) was utilized for statistical analysis. A two-sided $P < 0.05$ was considered as statistical significance, and 95% confidence intervals (CIs) were calculated.

RESULTS

Patient characteristics

All the 154 GBC patients in this study were treated at Peking Union Medical Hospital from January 2005 to May 2017. The median follow-up period was 17 mo. In total, 103 subjects died during the follow-up period, with an estimated median OS of 14.5 mo (range: 0.5-153.0 mo). The 1- and 2-year survival rates were 55.8% and 35.7%, respectively. The clinical data of all patients who met all criteria were analyzed. Among these patients, the median age at diagnosis was 64 years old (range: 29-85 years old), of whom, 98 (63.6%) were > 60 years old. Ninety-one (59.1%) patients were female. One hundred fifty (97.4%) patients were pathologically diagnosed with adenocarcinoma, three (1.9%) with adenosquamous cell carcinoma and one (0.6%) with papillary carcinoma. Ninety-four (61.0%) patients were histologically diagnosed with moderately or well-differentiated disease. Fifty-eight (37.7%) patients harbored a positive resection margin. According to the

Characteristic	Patients (*n* = 154)
Age (yr)	64 (29-85)
⩽ 60	56 (36.4)
> 60	98 (63.6)
Sex	
Male	63 (40.9)
Female	91 (59.1)
Cholecystolithiasis	
Absent	79 (51.3)
Present	75 (48.7)
Diabetes	
Absent	116 (75.3)
Present	38 (24.7)
Jaundice	
Absent	129 (83.8)
Present	25 (8.9)
Blood groups	
A	43 (27.9)
B	56 (36.4)
AB	9 (5.8)
O	46 (29.9)
Pathological types	
Adenosquamous carcinoma	3 (1.9)
Adenocarcinoma	150 (97.4)
Papillocarcinoma	1 (0.6)
Degree of differentiation	
Poor	60 (39.0)
Moderate-well	94 (61.0)
Resection margin status	
Negative	96 (62.3)
Positive	58 (37.7)
Maximum tumor diameter (cm)	3 (0.2-13)
⩽ 2.45	68 (44.2)
> 2.45	86 (55.8)
T stage	
Tis-T1a	10 (6.5)
T1b-T2b	29 (18.8)
T3	103 (66.9)
T4	12 (7.8)
N stage	
0	98 (63.6)
1	47 (30.5)
2	9 (5.8)
Distant metastasis	
Absent	142 (92.2)
Present	12 (7.8)
TNM stage	
0- I stage	16 (10.4)
ⅡA-ⅡB stage	16 (10.4)
ⅢA-ⅢB stage	92 (59.7)
ⅣA-ⅣB stage	30 (19.5)
CA199 (U/mL)	69.3 (0.6-10524)
⩽ 39	66 (42.9)
> 39	88 (57.1)
Fibrinogen concentration (g/L)	3.54 (1.71-7.47)
⩽ 3.47	75 (48.7)
> 3.47	79 (51.3)
Albumin levels (g/L)	41.0 (20.0-50.0)
⩽ 40.5	78 (50.6)
> 40.5	76 (49.4)
FAR	0.09 (0.04-0.25)
⩽ 0.08	71 (46.1)
> 0.08	83 (53.9)

FAR: Fibrinogen to albumin ratio.

TNM staging, most patients (59.7%) were classified as stage ⅢA-ⅢB. The detailed information of baseline characteristics of patients was shown in Table 1.

The optimal cut-off value of the preoperative fibrinogen concentration, albumin level and FAR for survival analysis
The ROC curves of OS were generated to validate the optimal cut-off values for the preoperative fibrinogen concentration, albumin level and FAR (Figure 1). The median plasma fibrinogen concentration in all patients was 3.54 g/L (range: 1.71-7.47 g/L) (Table 1). As shown in Figure 1A, the area under the curve (AUC) was recorded as 0.735 (95%CI: 0.654-0.816), and the optimal cut-off value of preoperative fibrinogen concentration for OS was 3.47 g/L, with the highest sensitivity and specificity of 0.709 and 0.721, respectively. Based on this cut-off, there were 75 patients (48.7%) with a fibrinogen concentration ⩽ 3.47 g/L, and 79 patients (51.3%) with a fibrinogen concentration > 3.47 g/L (Table 2).

The median serum albumin level in all patients was 41.0 g/L (range: 20.0-40.0 g/L) (Table 1). As shown in Figure 1B, the AUC was recorded as 0.648 (95%CI: 0.562-0.735), and the optimal cut-off value of the preoperative albumin level for OS was 40.5 g/L, with the highest sensitivity and specificity of 0.647 and 0.605, respectively. Based on this value, 76 patients (49.4%) had an albumin level ⩽ 40.5 g/L, and 78 patients (50.6%) had an albumin level > 40.5 g/L (Table 3).

The median FAR in all patients was 0.09 (range: 0.04-0.25) (Table 1). As shown in Figure 1C, the AUC was recorded as 0.783 (95%CI: 0.707-0.859), and the optimal cut-off value of the preoperative FAR for OS was 0.08, with the highest sensitivity and specificity of 0.779 and 0.765, respectively. Based on this value, 71 patients (46.1%) harbored a FAR value ⩽ 0.08, and 83 patients (53.9%) had a FAR value > 0.08 (Table 4).

Correlations of the preoperative fibrinogen concentration, albumin level and FAR with clinicopathological factors
As shown in Table 2, based on the optimal cut-off value for the preoperative fibrinogen concentration, all patients could be divided into the low-value group (⩽ 3.47 g/L) or the high-value group (> 3.47 g/L). Higher preoperative fibrinogen concentration was significantly correlated with jaundice (*P* = 0.003), degree of differentiation (*P* = 0.048), resection margin (*P* = 0.003), T stage (*P* < 0.001), TNM stage (*P* = 0.011), CA199 level (*P* = 0.005) as well as FAR (*P* < 0.001). However, there were no significant associations of the preoperative fibrinogen concentration with age, gender, cholecystolithiasis, diabetes, ABO blood group, pathological type, tumor size, N stage, distant metastasis or albumin level (*P* > 0.05). The survival curve stratified by the fibrinogen concentration indicated that GBC subjects with a fibrinogen concentration > 3.47 g/L had shorter OS than those with a fibrinogen concentration ⩽ 3.47 g/L (Figure 2A).
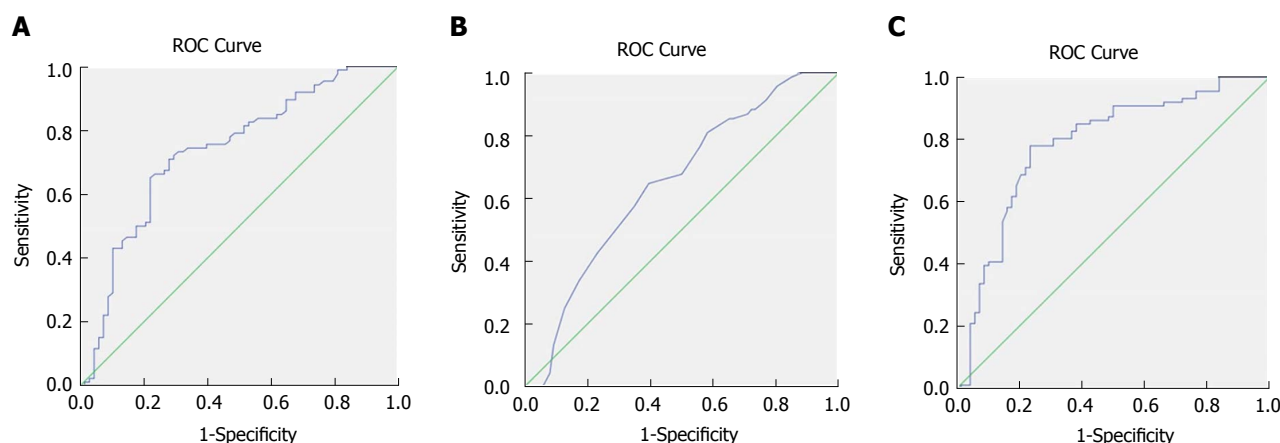
Figure 1 Receiver operating characteristics curve analysis based on fibrinogen (A), albumin (B), and fibrinogen to albumin ratio (C) for overall survival. A: The area under the ROC curve (AUC) indicates the diagnostic power of preoperative plasma fibrinogen concentration. In this model, the optimum cut-off point for fibrinogen concentration was 3.47 g/L, AUC was 0.735 (95%CI: 0.654-0.816), with a sensitivity of 0.709 and a specificity of 0.721 by the Youden index; B: The AUC indicates the diagnostic power of preoperative plasma albumin level. In this model, the optimum cut-off point for albumin level was 40.5 g/L, AUC was 0.648 (95%CI: 0.562-0.735), with a sensitivity of 0.647 and a specificity of 0.605 by the Youden index; C: The AUC indicates the diagnostic power of preoperative FAR. In this model, the optimum cut-off point for FAR was 0.08, AUC was 0.783 (95%CI: 0.707-0.859), with a sensitivity of 0.779 and a specificity of 0.765 by the Youden index. ROC: Receiver operating characteristics curve.

As shown in Table 3, based on the optimal cut-off value for the preoperative albumin level, all patients could be categorized into the low-value group ($\leqslant$ 40.5 g/L) or high-value group (> 40.5 g/L). Higher preoperative albumin levels were significantly associated with jaundice ($P < 0.001$), ABO blood group ($P = 0.046$), degree of differentiation ($P = 0.047$), resection margin status ($P = 0.008$), T stage ($P = 0.021$), TNM stage ($P = 0.007$), CA199 levels ($P = 0.006$) as well as FAR ($P < 0.001$). The survival curve stratified by the albumin level showed that GBC patients with an albumin level > 40.5 g/L had longer OS than those with an albumin level $\leqslant$ 40.5 g/L (Figure 2B).

As shown in Table 4, based on the optimal cut-off value for the preoperative FAR, all patients could be grouped into the low-value group ($\leqslant$ 0.08) or high-value group (> 0.08). A higher preoperative FAR was significantly correlated with age ($P = 0.045$), jaundice ($P < 0.001$), degree of differentiation ($P = 0.002$), resection margin status ($P < 0.001$), T stage ($P < 0.001$), TNM stage ($P < 0.001$), CA199 level ($P < 0.001$) as well as albumin level ($P < 0.001$). The survival curve stratified by the FAR showed that GBC patients with a FAR > 0.08 harbored worse OS compared to those with a FAR $\leqslant$ 0.08 (Figure 2C).

Univariate and multivariate analysis results
Univariate and multivariate analyses for OS prediction in GBC patients are shown in Tables 5 and 6. In the univariate Cox analysis, jaundice (HR: 2.598, 95%CI: 1.644-4.106, $P < 0.001$), degree of differentiation (HR: 1.527, 95%CI: 1.031-2.261, $P = 0.035$), resection margin status (HR: 3.683, 95%CI: 2.468-5.496, $P < 0.001$), T stage ($P < 0.001$), N stage ($P < 0.001$), distant metastasis (HR: 2.550, 95%CI: 1.388-4.684, $P = 0.003$), TNM stage ($P < 0.001$), CA199 level (HR: 3.125, 95%CI: 2.010-4.858, $P < 0.001$), fibrinogen

concentration (HR: 2.795, 95%CI: 1.853-4.214, $P < 0.001$), albumin level (HR: 0.391, 95%CI: 0.259-0.590, $P < 0.001$) and FAR (HR: 4.626, 95%CI: 2.987-7.165, $P < 0.001$) were significant prognostic factors for OS in GBC patients (Table 5), whereas age, gender, cholecystolithiasis, diabetes, ABO blood group, pathological type and maximal tumor diameter were not significant predictors of OS ($P > 0.05$; Table 5). In the multivariate Cox regression analysis, resection margin status (HR: 2.343, 95%CI: 1.532-3.581, $P < 0.001$, TNM stage ($P = 0.035$), and FAR (HR: 2.813, 95%CI: 1.765-4.484, $P < 0.001$) were revealed as independent risk factors for poor OS in GBC patients, and the albumin level (HR: 0.595, 95%CI: 0.385-0.921, $P = 0.020$) was correlated with favorable OS in patients with GBC (Table 6).

Although the multivariate analysis showed that both FAR and albumin level were independent risk factors for the prognosis of GBC patients (Table 6), the AUC of FAR (0.783) was greater than that (0.648) of the albumin level (Figure 1B and C), indicating that the prognostic value of FAR was more powerful than that of the albumin level.

DISCUSSION

In this study, we demonstrate FAR is a significantly independent prognostic indicator for GBC. To our knowledge, it is the first research concerning the prognostic significance of FAR in patients with GBC. Although both FAR and serum albumin level were revealed to be significant prognostic indicators, the AUC of FAR was greater than that of the serum albumin level, and the P value of FAR was smaller than that of the serum albumin level.

In our study, a greater FAR was found to be correlated with a series of important clinicopathological indicators

Table 2 Correlation between fibrinogen concentration and clinicopathological characteristics in gallbladder cancer patients *n* (%)

| Characteristics | Fibrinogen concentration | | *P* value |
	≤ 3.47 g/L (*n* = 75)	> 3.47 g/L (*n* = 79)	
Age (yr)			
≤ 60	31 (20.1)	25 (16.2)	0.243
> 60	44 (28.6)	54 (35.1)	
Sex			
Male	33 (21.4)	30 (19.5)	0.513
Female	42 (27.3)	49 (31.8)	
Cholecystolithiasis			
Absent	38 (24.7)	41 (26.6)	0.878
Present	37 (24.0)	38 (24.7)	
Diabetes			
Absent	57 (37.0)	59 (38.3)	0.850
Present	18 (11.7)	20 (13.0)	
Jaundice			
Absent	68 (44.2)	61 (39.6)	0.029
Present	7 (4.5)	18 (11.7)	
Blood groups			
A	19 (12.3)	24 (15.6)	0.145
B	33 (21.4)	23 (14.9)	
AB	2 (1.3)	7 (4.5)	
O	21 (13.6)	25 (16.2)	
Pathological types			
Adenosquamous carcinoma	0 (0)	3 (1.9)	0.142
Adenocarcinoma	75 (48.7)	75 (48.7)	
Papillocarcinoma	0 (0)	1 (0.6)	
Degree of differentiation			
Poor	23 (14.9)	37 (24.0)	0.048
Moderate-well	52 (33.8)	42 (27.3)	
Resection margin status			
Negative	56 (36.4)	40 (26.4)	0.003
Positive	19 (12.3)	39 (25.3)	
Maximum tumor diameter (cm)			
≤ 2.45	34 (22.1)	34 (22.1)	0.871
> 2.45	41 (26.6)	45 (29.2)	
T stage			
Tis-T1a	8 (5.2)	2 (1.3)	< 0.001
T1b-T2b	22 (14.3)	7 (4.5)	
T3	43 (27.9)	60 (39.0)	
T4	2 (1.3)	10 (6.5)	
N stage			
N0	50 (32.5)	48 (31.2)	0.748
N1	21 (13.6)	26 (16.9)	
N2	4 (2.6)	5 (3.2)	
Distant metastasis			
Absent	69 (44.8)	73 (47.4)	0.925
Present	6 (3.9)	6 (3.9)	
TNM stage			
0-Ⅰ stage	12 (7.8)	4 (2.6)	0.011
ⅡA-ⅡB stage	12 (7.8)	4 (2.6)	
ⅢA-ⅢB stage	39 (25.3)	53 (34.4)	
ⅣA-ⅣB stage	12 (7.8)	18 (11.7)	
CA199 (U/mL)			
≤ 39	41 (26.6)	25 (16.2)	0.005
> 39	34 (22.1)	54 (35.1)	
Albumin levels (g/L)			
≤ 40.5	32 (20.8)	44 (28.6)	0.111
> 40.5	43 (27.9)	35 (22.7)	
FAR			
≤ 0.08	59 (38.3)	12 (7.8)	< 0.001
> 0.08	16 (10.4)	67 (43.5)	

FAR: Fibrinogen to albumin ratio.

Table 3 Correlation between albumin levels and clinicopathological characteristics in gallbladder cancer patients *n* (%)

| Characteristics | Albumin levels | | *P* value |
	≤ 40.5g/L (*n* = 76)	> 40.5 g/L (*n* = 78)	
Age (yr)			
≤ 60	22 (14.3)	34 (22.1)	0.067
> 60	54 (35.1)	44 (28.6)	
Sex			
Male	28 (18.2)	35 (22.7)	0.330
Female	48 (31.2)	43 (27.9)	
Cholecystolithiasis			
Absent	34 (22.1)	45 (29.2)	0.147
Present	42 (27.3)	33 (21.4)	
Diabetes			
Absent	53 (34.4)	63 (40.9)	0.136
Present	23 (14.9)	15 (9.7)	
Jaundice			
Absent	54 (35.1)	75 (48.7)	< 0.001
Present	22 (14.3)	3 (1.9)	
Blood groups			
A	20 (13.0)	23 (14.9)	0.046
B	34 (22.1)	22 (14.3)	
AB	6 (7.9)	3 (3.8)	
O	16 (21.1)	30 (19.5)	
Pathological types			
Adenosquamous carcinoma	0 (0)	3 (1.9)	0.137
Adenocarcinoma	75 (48.7)	75 (48.7)	
Papillocarcinoma	1 (0.6)	0 (0.0)	
Degree of differentiation			
Poor	36 (23.4)	24 (15.6)	0.047
Moderate-well	40 (26.0)	54 (35.1)	
Resection margin status			
Negative	39 (25.3)	57 (37.0)	0.008
Positive	37 (24.0)	21 (13.6)	
Maximum tumor diameter (cm)			
≤ 2.45	36 (23.4)	32 (20.8)	0.516
> 2.45	40 (26.0)	46 (29.9)	
T stage			
Tis-T1a	2 (1.3)	8 (5.2)	0.021
T1b-T2b	9 (5.8)	20 (13.0)	
T3	58 (37.7)	45 (29.2)	
T4	7 (4.5)	5 (3.2)	
N stage			
N0	45 (29.2)	53 (34.4)	0.403
N1	25 (16.2)	22 (14.3)	
N2	6 (3.9)	3 (1.9)	
Distant metastasis			
Absent	67 (43.5)	75 (48.7)	0.077
Present	9 (5.8)	3 (1.9)	
TNM stage			
0-Ⅰ stage	3 (1.9)	13 (8.4)	0.007
ⅡA-ⅡB stage	6 (3.9)	10 (6.5)	
ⅢA-ⅢB stage	46 (29.9)	46 (29.9)	
ⅣA-ⅣB stage	21 (13.6)	9 (5.8)	
CA199 (U/mL)			
≤ 39	24 (15.6)	42 (27.3)	0.006
> 39	52 (33.8)	36 (23.4)	
Fibrinogen concentration (g/L)			0.111
≤ 3.47g/L	32 (20.8)	43 (27.9)	
> 3.47 g/L	44 (28.6)	35 (22.7)	
FAR			
≤ 0.08	21 (13.6)	50 (32.5)	< 0.001
> 0.08	55 (35.7)	28 (18.2)	

FAR: Fibrinogen to albumin ratio.

Table 4 Correlation between FAR and clinicopathological characteristics in gallbladder cancer patients *n* (%)

Characteristics	FAR		*P* value
	≤ 0.08 (*n* = 71)	> 0.08 (*n* = 83)	
Age (yr)			
≤ 60	32 (20.8)	24 (15.6)	0.045
> 60	39 (25.3)	59 (38.3)	
Sex			
Male	30 (19.5)	33 (21.4)	0.870
Female	41 (26.6)	50 (32.5)	
Cholecystolithiasis			
Absent	37 (24.0)	42 (27.3)	0.873
Present	34 (22.1)	41 (26.6)	
Diabetes			
Absent	56 (36.4)	60 (39.0)	0.357
Present	15 (9.7)	23 (14.9)	
Jaundice			
Absent	67 (43.5)	62 (40.3)	< 0.001
Present	4 (2.6)	21 (13.6)	
Blood groups			
A	22 (14.3)	21 (13.6)	0.148
B	28 (18.2)	28 (18.2)	
AB	1 (0.6)	8 (5.2)	
O	20 (13.0)	26 (16.9)	
Pathological types			
Adenosquamous carcinoma	0 (0)	3 (1.9)	0.173
Adenocarcinoma	71 (46.1)	79 (51.3)	
Papillocarcinoma	0 (0)	1 (0.6)	
Degree of differentiation			
Poor	18 (11.7)	42 (27.3)	0.002
Moderate-well	53 (34.4)	41 (26.6)	
Resection margin status			
Negative	55 (35.7)	41 (26.6)	< 0.001
Positive	16 (10.4)	42 (27.3)	
Maximum tumor diameter (cm)			
≤ 2.45	37 (24.0)	31 (20.1)	0.075
> 2.45	34 (22.1)	52 (33.8)	
T stage			
Tis-T1a	8 (5.2)	2 (1.3)	< 0.001
T1b-T2b	24 (15.6)	5 (3.2)	
T3	36 (23.4)	67 (43.5)	
T4	3 (1.9)	9 (5.8)	
N stage			
N0	48 (31.2)	50 (32.5)	0.623
N1	19 (12.3)	28 (18.2)	
N2	4 (2.6)	5 (3.2)	
Distant metastasis			
Absent	68 (44.2)	74 (48.1)	0.145
Present	3 (1.9)	9 (5.8)	
TNM stage			
0- Ⅰ stage	14 (9.1)	2 (1.3)	< 0.001
ⅡA-ⅡB stage	14 (9.1)	2 (1.3)	
ⅢA-ⅢB stage	35 (22.7)	57 (37.0)	
ⅣA-ⅣB stage	8 (5.2)	22 (14.3)	
CA199 (U/mL)			
≤ 39	43 (27.9)	23 (14.9)	< 0.001
> 39	28 (18.2)	60 (39.0)	
Fibrinogen concentration (g/L)			
≤ 3.47g/L	59 (38.3)	16 (10.4)	< 0.001
> 3.47 g/L	12 (7.8)	67 (43.5)	
Albumin levels (g/L)			
≤ 40.5g/L	21 (13.6)	55 (35.7)	< 0.001
> 40.5 g/L	50 (32.5)	28 (18.2)	

of GBC patients, such as resection margin status, TNM stage and albumin level, which were independent risk

factors for OS in GBC, indicating that an elevated FAR might be associated with aggressiveness and systemic progression of GBC.

Relatively few previous studies have probed into the prognostic significance of FAR in patients with malignant tumors. To date, there are only two studies performed in the context of breast cancer[23] and ESCC[22]. In line with our findings, the optimal cut-off value of FAR for ESCC patients was also 0.08. However, the optimal cut-off value of FAR in breast cancer was 0.071, which is slightly lower than that in ESCC patients and in GBC subjects in our study. Together, the inconsistent findings indicate that the optimal FAR cut-off value varies in different malignancies. Although the exact cause and underlying mechanism of these differences remain unknown, they might be related to the different biological behaviors of different tumors and gender-associated hormone difference. Hence, more studies are needed to further verify these conclusions.

Inconsistent with these previous two studies, our study indicates that the preoperative albumin level is also an independent risk factor for OS in GBC patients, and an elevated albumin level is a favorable prognostic factor for GBC patients. Several studies have demonstrated that lower serum albumin levels could lead to deteriorated diseases and a greater risk of poor prognosis in patients with gastric cancer[26], ovarian cancer[27] and upper urinary tract urothelial carcinoma[12]. However, to our knowledge, it is the first study to assess the prognostic significance of preoperative serum albumin in GBC.

Accumulating studies have demonstrated the effect of activated coagulation with fibrinolysis, malnutrition and inflammation during carcinogenesis, cancer progression and metastasis[28-31]. Although the prognostic value of the preoperative FAR has been established in patients with malignant tumors[22,23], the real mechanisms underlying this association remains largely undefined. Our observations are supported by several previous experimental and clinical researches. As a P-globulin and pro-inflammatory protein, fibrinogen can be synthesized by malignant tumor cells apart from hepatic cells, which participates in extracellular matrix (ECM) formation[32-34]. Fibrinogen can promote tumor progression *via* regulation of tumor cell growth by binding to vascular endothelial growth factor (VEGF) as well as platelet-derived growth factor (PDGF)[33-35]. An experimental study has demonstrated that fibrinogen can induce epithelial-mesenchymal transition (EMT) to enhance the migration and invasion ability of tumor cells via modulation of the expression of vimentin and E-cadherin[36]. Another experimental study performed in fibrinogen-deficient mice indicates that fibrinogen-free internal environment can suppress the spread of tumor cells and the subsequent establishment of micro-metastases[37]. A previous study[38] also showed that fibrinogen could facilitate tumor cell metastasis by suppressing natural killer (NK) cell-mediated apoptosis. Fibrinogen has
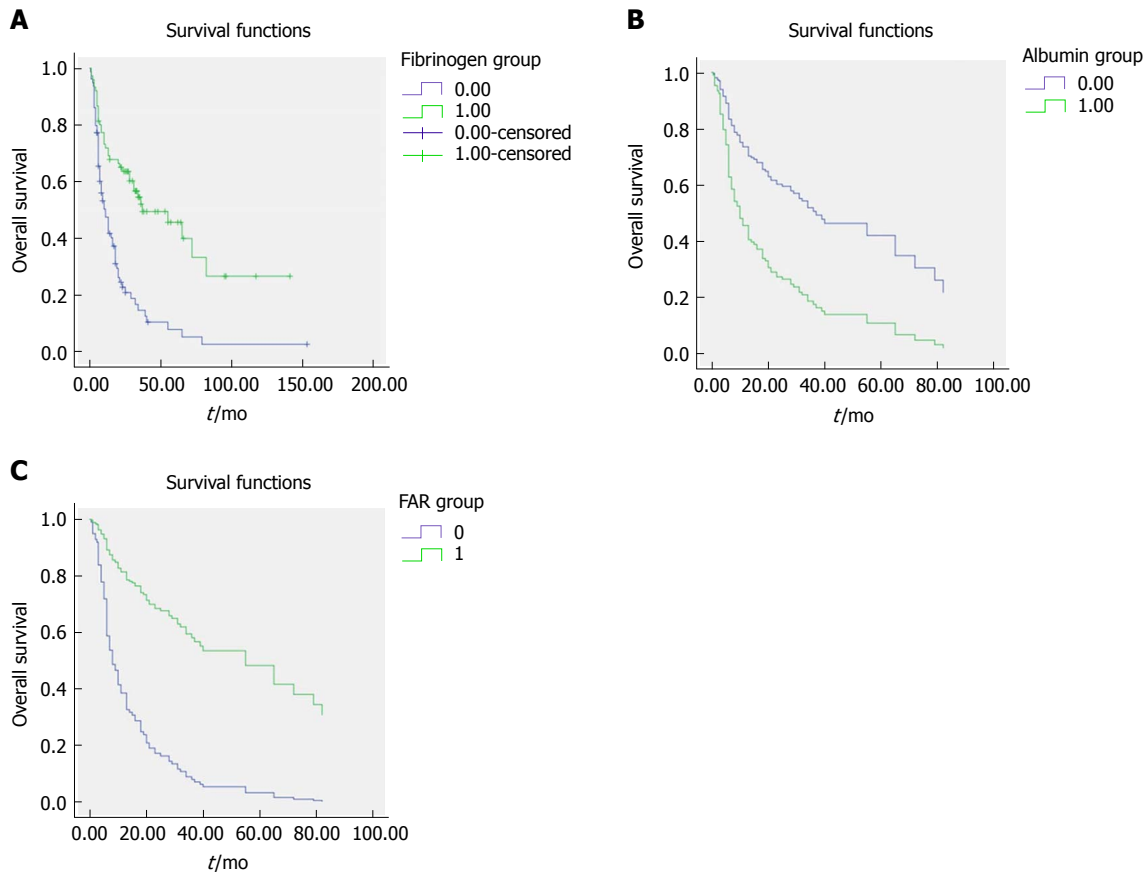
Figure 2 Survival curve according to the presence of preoperative fibrinogen concentration (A), albumin level (B), and fibrinogen to albumin ratio (C).
A: Data compares fibrinogen concentration > 3.47 g/L *vs* ⩽ 3.47 g/L group ($P < 0.05$). The number 1 for ⩽ 3.47 g/L group, number 2 for > 3.47g/L group; B: Data compares albumin level > 40.5 g/L *vs* ⩽ 40.5 g/L group ($P < 0.05$). The number 0 for albumin level > 40.5 g/L group, number 1 for albumin level ⩽ 40.5 g/L group; C: Data compares FAR > 0.08 *vs* ⩽ 0.08 group ($P < 0.05$). The number 1 for FAR > 0.08 group, number 0 for FAR ⩽ 0.08 g/L group. FAR: Fibrinogen to albumin ratio.

also been demonstrated to be critically involved in the tumorigenesis and tumor progression *via* aggravation of cell proliferation, suppression of apoptosis and stimulation of angiogenesis as well as hematogenous metastasis[33,34,39-41]. The albumin level can not only reflect the malnutrition status of host, but also implicate the existence of inflammation. Malnutrition is commonly detected in cancer patients, which might lead to multiple negative outcomes, including compromised immune function, insensitive therapeutic response as well as reduced OS[42]. As part of the SIR to tumor or from tumor itself, inflammatory mediators are secreted, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 as well as acute-phase reactants. IL-6 has been suggested to modulate VEGF secretion from glioblastoma cells, and the latter can result in vascular permeability, contributing to declined serum albumin levels[43,44]. Proinflammatory cytokines, such as TNF-α, IL-1 and IL-6 can downregulate the hepatic synthesis of albumin[45-48]. Therefore, albumin level might be used to reflect tumor prognosis. Taken together, FAR could be considered as a prognostic factor for GBC patients.

There are certain limitations in this study. To begin with, this study was a retrospective, small-sample, single-center one, hence, there might be a selection bias. Secondly, due to the small sample size, we were unable to perform further subgroup analysis according to different models, such as the TNM stage model, treatment model and distant metastasis model. Thirdly, our findings lacked external verification, which requires further investigation. Fourth, although the cut-off values were calculated by ROC curves, they were based on a relatively small sample; as such, other cut-off values may be more accurate in the case of increased sample size. In this study, we mainly focused on the prognostic significance of the preoperative FAR, while changes in the postoperative FAR have not been studied; thus, the prognostic value of the postoperative FAR was not assessed. Therefore, more well-designed, prospective and large-sample multi-center studies are warranted to further verify the present conclusions.

In conclusion, the preoperative FAR is a significant and powerful negative prognostic indicator for OS in GBC patients, and the preoperative serum albumin level is a favorable prognostic factor for OS in GBC patients, and the predictive power of FAR is greater than that of the albumin level. As a simple, convenient and cost-effective indicator, FAR, defined as the fibrinogen-to-albumin ratio, could easily be applied in the clinical setting *via* routine preoperative laboratory tests to predict the

Table 5　Univariate analysis of overall survival in gallbladder cancer patients

Characteristics	HR (95%CI)	*P* value
Age (yr)	1.473 (0.973-2.230)	0.067
≤ 60		
> 60		
Sex	0.995 (0.670-1.477)	0.981
Male		
Female		
Cholecystolithiasis	1.198 (0.814-1.764)	0.360
Absent		
Present		
Diabetes	1.028 (0.651-1.623)	0.906
Absent		
Present		
Jaundice	2.598 (1.644-4.106)	< 0.001
Absent		
Present		
Blood groups	-	0.113
A		
B		
AB		
O		
Pathological types	-	0.165
Adenosquamous carcinoma		
Adenocarcinoma		
Papillocarcinoma		
Degree of differentiation	1.527 (1.031-2.261)	0.035
Poor		
Moderate-well		
Resection margin status	3.683 (2.468-5.496)	< 0.001
Negative		
Positive		
Maximum tumor diameter (cm)	1.101 (0.744-1.630)	0.631
≤ 2.45		
> 2.45		
T stage	-	< 0.001
Tis-T1a		
T1b-T2b		
T3		
T4		
N stage	-	< 0.001
0		
1		
2		
Distant metastasis	2.550 (1.388-4.684)	0.003
Absent		
Present		
TNM stage	-	< 0.001
0- I stage		
ⅡA-ⅡB stage		
ⅢA-ⅢB stage		
ⅣA-ⅣB stage		
CA199 (U/mL)	3.125 (2.010-4.858)	< 0.001
≤ 39		
> 39		
Fibrinogen concentration (g/L)	2.795 (1.853-4.214)	< 0.001
≤ 3.47		
> 3.47		
Albumin levels (g/L)	0.391(0.259-0.590)	< 0.001
≤ 40.5		
> 40.5		
FAR	4.626(2.987-7.165)	< 0.001
≤ 0.08		
> 0.08		

FAR: Fibrinogen to albumin ratio; HR: Hazard ratio; CI: Confidence interval.

Table 6　Multivariate analysis for overall survival in gallbladder cancer patients

Characteristics	HR (95%CI)	Wald	*P* value
Resection margin status	2.343 (1.532-3.581)		< 0.001
Negative			
Positive			
TNM stage		8.595	0.035
ⅡA-ⅡB stage/0-1stage	1.209 (0.287-5.095)	0.067	0.796
ⅢA-ⅢB stage/0-1 stage	3.401 (1.033-11.202)	4.051	0.044
ⅣA-ⅣB stage/0-1 stage	4.014 (1.142-14.107)	4.696	0.030
FAR	2.813 (1.765-4.484)		< 0.001
≤ 0.08			
> 0.08			
Albumin levels (g/L)	0.595 (0.385-0.921)		0.020
≤ 40.5			
> 40.5			

FAR: Fibrinogen to albumin ratio; HR: Hazard ratio; CI: Confidence interval.

prognosis of GBC patients. However, more related studies are warranted to validate these conclusions.

ARTICLE HIGHLIGHTS

Research background
Although gallbladder cancer (GBC) is a relatively rare hepato-biliary malignancy with a low incidence, it is generally insidious and progresses rapidly. Most GBC patients are diagnosed at an advanced stage, losing the chance of surgical intervention, which is considered to yield an optimal therapeutic effect. Despite the great advance in surgical techniques in recent years, the prognosis remains very poor. Therefore, it is urgent to explore a clinically simple, convenient and cost-effective prognostic indicator to detect and identify high-risk patients with GBC, on whom, appropriate surgical treatment can be performed as soon as possible. In recent years, a variety of studies have shown that the increased plasma fibrinogen concentration representing coagulation function of the body and the declined plasma albumin concentration indicating nutrient state of the body are independent risk factors for poor prognosis of malignant tumor patients. Integrating the results of studies on fibrinogen-to-albumin ratio (FAR) in the prognosis of patients with esophageal cancer and breast cancer, we naturally speculate that FAR might be significantly more effective than single elevated plasma fibrinogen concentration or reduced plasma albumin concentration in predicting the prognosis of GBC patients.

Research motivation
Hence, the present was mainly designed to determine and verify the role of high FAR in the prognosis of surgically-treated GBC patients. We aimed to detect a simple, convenient and cost-effective prognostic biomarker for GBC patients undergoing surgical treatment, which could facilitate the selection and identification of GBC patients suitable for surgical resection for clinical surgeons. Notably, this would be beneficial to both surgeons and GBC patients. Our findings would provide clinical evidence and research directions for other large-scale, multi-center randomized controlled trials in the future.

Research objectives
The main objective of our study was to determine whether high preoperative FAR was an independent risk factors for postoperative survival in GBC patients. As a result, we demonstrated that high preoperative plasma FAR value and low preoperative plasma albumin concentration were independent risk factors for poor post-operative prognosis of GBC patients. In addition, the prognostic effect of high preoperative FAR value was significantly stronger than the low preoperative plasma albumin concentration. Therefore, these above-described outcomes provided not only clinical direction for further clinical validation or relevant studies, but also clinical data for further researches concerning the

underlying mechanisms.

Research methods

First, the present study was a clinical retrospective one. A prearranged EXCEL data collection table was utilized to collect and organize the various variables, including epidemiological data, clinicopathological characteristics, and research-related target data. Moreover, the receiver operating characteristic (ROC) curve was used to obtain the optimal cut-off values for fibrinogen, albumin, and FAR. Continuous variables in normal distribution were shown as mean ± SD, and continuous variables without normal distribution were expressed as medians (range: minimum-maximum). Categorical variables were expressed as percentages or frequencies. Variables from the EXCEL table were further imported into the SPSS 24.0 statistical software for statistical analysis. Of note, the statistical methods used in our study were different from those used in previous studies of survival analysis regarding the prognosis of cancer patients. To begin with, the ROC curve was used to identify the optimal cut-off value of fibrinogen, albumin, and FAR in this study, which was more reasonable and more scientific than the traditional methods, which used the mean value of the targeted or identified biomarkers based on previous studies. It was because the cut-off value identified by this method was significantly associated with the overall survival of the targeted population. Secondly, most of the previous studies on postoperative prognosis of GBC patients only focused on single index, such as plasma fibrinogen or plasma albumin. However, in this study, we used the plasma FAR, representing the division of high fibrinogen and low albumin, which contributed to the more significant prognostic effect of the index, and effectively inhibited the influence of confounding factors. Together, the method was more scientific and harbored higher statistical efficiency.

Research results

In this study, we demonstrated that high preoperative plasma FAR and low preoperative plasma albumin concentration were independent risk factors for poor postoperative outcome in GBC patients. To the best of our knowledge, this is the first study indicating that high preoperative plasma FAR is an unfavorable prognostic biomarker for GBC patients undergoing surgical intervention. Additionally, it also verifies the role of low preoperative plasma albumin in predicting the worse prognosis of GBC patients receiving surgery. Nevertheless, our study is a retrospective study but not a prospective study, which might lead to a systematic bias. Moreover, the sample size in our study is relatively small, and it is a single-center study, and these defects would attenuate the statistical effectiveness of our conclusions. Nevertheless, the study was conducted in China, which did not include GBC patients from other ethnic groups and countries, thereby affecting the clinical applicability and generalizability of the results. Therefore, more multiple-center, large-scale prospective studies enrolling GBC patients from different races and countries are necessary to further verify the conclusions of this study.

Research conclusions

At present, accumulating studies have confirmed that high preoperative plasma fibrinogen concentration and low preoperative plasma albumin concentration are independent risk factors for poor prognosis of GBC patients. In addition, some studies have further validated that high preoperative plasma FAR is an independent risk factor for poor prognosis of patients with esophageal cancer and breast cancer, and its predictive ability is significantly more potent than that of single biomarkers, such as high plasma fibrinogen and low plasma albumin. Therefore, we naturally speculated that FAR, representing the body's coagulation function and the body's nutritional status, might be an independent risk factor for predicting postoperative adverse outcomes of GBC patients, which has been confirmed in our study. Our study was the first to reveal the prognostic effect of FAR in GBC patients, and we also used the ROC curve as a novel method to identify the optimal cut-off value for the prognostic index studied. The potential mechanism for our conclusion might be indicated as follows: fibrinogen, as a coagulation factor, was associated with the growth, progression and metastasis of cancer cells, while albumin was correlated with the nutrient status and immune function of the body. Therefore, the high preoperative plasma fibrinogen and low preoperative albumin are both unfavorable prognostic factors for GBC patients. The FAR can enhance and magnify the prognostic effect of the single index such as fibrinogen and albumin. Collectively, our research provides a simple, convenient and cost-effective prognostic indicator to help clinicians to more efficiently screen and identify high-risk GBC patients in clinical practice, and to facilitate patients to adopt better surgical methods and optimal follow-up strategy in the future.

Research perspectives

In the present study, it is indicated that the plasma FAR, incorporating two biomarkers, harbors a significantly better prognostic impact on surgically-treated GBC patients compared to a single prognostic indicator, such as plasma albumin or plasma fibrinogen. In the future, more large-scale, multiple-center and prospective studies, including GBC patients from other races and countries, should be conducted to further investigate and verify the conclusion derived from our study. Additionally, more basic experiments exploring the potential mechanisms are also necessary in the future.

REFERENCES

1   **Zhu AX**, Hong TS, Hezel AF, Kooby DA. Current management of gallbladder carcinoma. *Oncologist* 2010; **15**: 168-181 [PMID: 20147507 DOI: 10.1634/theoncologist.2009-0302]

2   **Paliogiannis P**, Scognamillo F, Attene F, Marrosu A, Trignano E, Tedde L, Delogu D, Trignano M. Preneoplastic and neoplastic gallbladder lesions occasionally discovered after elective videocholecystectomy for benign disease. A single centre experience and literature review. *Ann Ital Chir* 2013; **84**: 281-285 [PMID: 23047610]

3   **Misra S**, Chaturvedi A, Misra NC, Sharma ID. Carcinoma of the gallbladder. *Lancet Oncol* 2003; **4**: 167-176 [PMID: 12623362 DOI: 10.1016/S1470-2045(03)01021-0]

4   **Duffy A**, Capanu M, Abou-Alfa GK, Huitzil D, Jarnagin W, Fong Y, D'Angelica M, Dematteo RP, Blumgart LH, O'Reilly EM. Gallbladder cancer (GBC): 10-year experience at Memorial Sloan-Kettering Cancer Centre (MSKCC). *J Surg Oncol* 2008; **98**: 485-489 [PMID: 18802958 DOI: 10.1002/jso.21141]

5   **Lai EC**, Lau WY. Aggressive surgical resection for carcinoma of gallbladder. *ANZ J Surg* 2005; **75**: 441-444 [PMID: 15943734 DOI: 10.1111/j.1445-2197.2005.03401.x]

6   **Bartlett DL**, Fong Y, Fortner JG, Brennan MF, Blumgart LH. Long-term results after resection for gallbladder cancer. Implications for staging and management. *Ann Surg* 1996; **224**: 639-646 [PMID: 8916879]

7   **Forrest LM**, McMillan DC, McArdle CS, Angerson WJ, Dunlop DJ. Comparison of an inflammation-based prognostic score (GPS) with performance status (ECOG) in patients receiving platinum-based chemotherapy for inoperable non-small-cell lung cancer. *Br J Cancer* 2004; **90**: 1704-1706 [PMID: 15150622 DOI: 10.1038/sj.bjc.6601789]

8   **Ahn HS**, Lee HJ, Yoo MW, Kim SG, Im JP, Kim SH, Kim WH, Lee KU, Yang HK. Diagnostic accuracy of T and N stages with endoscopy, stomach protocol CT, and endoscopic ultrasonography in early gastric cancer. *J Surg Oncol* 2009; **99**: 20-27 [PMID: 18937292 DOI: 10.1002/jso.21170]

9   **Lee HH**, Lim CH, Park JM, Cho YK, Song KY, Jeon HM, Park CH. Low accuracy of endoscopic ultrasonography for detailed T staging in gastric cancer. *World J Surg Oncol* 2012; **10**: 190 [PMID: 22978534 DOI: 10.1186/1477-7819-10-190]

10 **Ando M**, Ando Y, Hasegawa Y, Shimokata K, Minami H, Wakai K, Ohno Y, Sakai S. Prognostic value of performance status assessed by patients themselves, nurses, and oncologists in advanced non-small cell lung cancer. *Br J Cancer* 2001; **85**: 1634-1639 [PMID: 11742480 DOI: 10.1054/bjoc.2001.2162]

11 **Rajan R**, Poniecka A, Smith TL, Yang Y, Frye D, Pusztai L, Fiterman DJ, Gal-Gombos E, Whitman G, Rouzier R, Green M, Kuerer H, Buzdar AU, Hortobagyi GN, Symmans WF. Change in tumor cellularity of breast carcinoma after neoadjuvant chemotherapy as a variable in the pathologic assessment of response. *Cancer* 2004; **100**: 1365-1373 [PMID: 15042669 DOI: 10.1002/cncr.20134]

12 **Ku JH**, Kim M, Choi WS, Kwak C, Kim HH. Preoperative serum albumin as a prognostic factor in patients with upper urinary tract urothelial carcinoma. *Int Braz J Urol* 2014; **40**: 753-762 [PMID:

25615244 DOI: 10.1590/S1677-5538.IBJU.2014.06.06]

13   **Perisanidis C**, Psyrri A, Cohen EE, Engelmann J, Heinze G, Perisanidis B, Stift A, Filipits M, Kornek G, Nkenke E. Prognostic role of pretreatment plasma fibrinogen in patients with solid tumors: A systematic review and meta-analysis. *Cancer Treat Rev* 2015; **41**: 960-970 [PMID: 26604093 DOI: 10.1016/j.ctrv.2015.10.002]

14   **Son HJ**, Park JW, Chang HJ, Kim DY, Kim BC, Kim SY, Park SC, Choi HS, Oh JH. Preoperative plasma hyperfibrinogenemia is predictive of poor prognosis in patients with nonmetastatic colon cancer. *Ann Surg Oncol* 2013; **20**: 2908-2913 [PMID: 23612884 DOI: 10.1245/s10434-013-2968-8]

15   **Bairey O**, Shacham-Abulafia A, Shpilberg O, Gurion R. Serum albumin level at diagnosis of diffuse large B-cell lymphoma: an important simple prognostic factor. *Hematol Oncol* 2016; **34**: 184-192 [PMID: 26052918 DOI: 10.1002/hon.2233]

16   **Gupta D**, Lis CG. Pretreatment serum albumin as a predictor of cancer survival: a systematic review of the epidemiological literature. *Nutr J* 2010; **9**: 69 [PMID: 21176210 DOI: 10.1186/1475-2891-9-69]

17   **Lee JH**, Hyun JH, Kim DY, Yoo BC, Park JW, Kim SY, Chang HJ, Kim BC, Kim TH, Oh JH, Sohn DK. The role of fibrinogen as a predictor in preoperative chemoradiation for rectal cancer. *Ann Surg Oncol* 2015; **22**: 209-215 [PMID: 25384698 DOI: 10.1245/s10434-014-3962-5]

18   **Zhao J**, Zhao M, Jin B, Yu P, Hu X, Teng Y, Zhang J, Luo Y, Zhang L, Zheng S, Zhou Q, Li H, Liu Y, Qu X. Tumor response and survival in patients with advanced non-small-cell lung cancer: the predictive value of chemotherapy-induced changes in fibrinogen. *BMC Cancer* 2012; **12**: 330 [PMID: 22852778 DOI: 10.1186/1471-2407-12-330]

19   **Pichler M**, Hutterer GC, Stojakovic T, Mannweiler S, Pummer K, Zigeuner R. High plasma fibrinogen level represents an independent negative prognostic factor regarding cancer-specific, metastasis-free, as well as overall survival in a European cohort of non-metastatic renal cell carcinoma patients. *Br J Cancer* 2013; **109**: 1123-1129 [PMID: 23922109 DOI: 10.1038/bjc.2013.443]

20   **Wen J**, Yang Y, Ye F, Huang X, Li S, Wang Q, Xie X. The preoperative plasma fibrinogen level is an independent prognostic factor for overall survival of breast cancer patients who underwent surgical treatment. *Breast* 2015; **24**: 745-750 [PMID: 26482138 DOI: 10.1016/j.breast.2015.09.007]

21   **Borg N**, Guilfoyle MR, Greenberg DC, Watts C, Thomson S. Serum albumin and survival in glioblastoma multiforme. *J Neurooncol* 2011; **105**: 77-81 [PMID: 21409514 DOI: 10.1007/s11060-011-0562-0]

22   **Tan Z**, Zhang M, Han Q, Wen J, Luo K, Lin P, Zhang L, Yang H, Fu J. A novel blood tool of cancer prognosis in esophageal squamous cell carcinoma: the Fibrinogen/Albumin Ratio. *J Cancer* 2017; **8**: 1025-1029 [PMID: 28529615 DOI: 10.7150/jca.16491]

23   **Hwang KT**, Chung JK, Roh EY, Kim J, Oh S, Kim YA, Rhu J, Kim S. Prognostic Influence of Preoperative Fibrinogen to Albumin Ratio for Breast Cancer. *J Breast Cancer* 2017; **20**: 254-263 [PMID: 28970851 DOI: 10.4048/jbc.2017.20.3.254]

24   **General Assembly of the World Medical Association**. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. *J Am Coll Dent* 2014; **81**: 14-18 [PMID: 25951678]

25   **CLAUSS A**. [Rapid physiological coagulation method in determination of fibrinogen]. *Acta Haematol* 1957; **17**: 237-246 [PMID: 13434757 DOI: 10.1159/000205234]

26   **Crumley AB**, Stuart RC, McKernan M, McMillan DC. Is hypoalbuminemia an independent prognostic factor in patients with gastric cancer? *World J Surg* 2010; **34**: 2393-2398 [PMID: 20602101 DOI: 10.1007/s00268-010-0641-y]

27   **Asher V**, Lee J, Bali A. Preoperative serum albumin is an independent prognostic predictor of survival in ovarian cancer. *Med Oncol* 2012; **29**: 2005-2009 [PMID: 21735143 DOI: 10.1007/s12032-011-0019-5]

28   **Unsal E**, Atalay F, Atikcan S, Yilmaz A. Prognostic significance of hemostatic parameters in patients with lung cancer. *Respir*

Med 2004; **98**: 93-98 [PMID: 14971870 DOI: 10.1016/j.rmed.2003.07.001]

29   **Torre LA**, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**: 87-108 [PMID: 25651787 DOI: 10.3322/caac.21262]

30   **Sun KY**, Xu JB, Chen SL, Yuan YJ, Wu H, Peng JJ, Chen CQ, Guo P, Hao YT, He YL. Novel immunological and nutritional-based prognostic index for gastric cancer. *World J Gastroenterol* 2015; **21**: 5961-5971 [PMID: 26019461 DOI: 10.3748/wjg.v21.i19.5961]

31   **Zitvogel L**, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat Rev Immunol* 2006; **6**: 715-727 [PMID: 16977338 DOI: 10.1038/nri1936]

32   **Simpson-Haidaris PJ**, Rybarczyk B. Tumors and fibrinogen. The role of fibrinogen as an extracellular matrix protein. *Ann N Y Acad Sci* 2001; **936**: 406-425 [PMID: 11460495 DOI: 10.1111/j.1749-6632.2001.tb03525.x]

33   **Sahni A**, Khorana AA, Baggs RB, Peng H, Francis CW. FGF-2 binding to fibrin(ogen) is required for augmented angiogenesis. *Blood* 2006; **107**: 126-131 [PMID: 16160009 DOI: 10.1182/blood-2005-06-2460]

34   **Sahni A**, Simpson-Haidaris PJ, Sahni SK, Vaday GG, Francis CW. Fibrinogen synthesized by cancer cells augments the proliferative effect of fibroblast growth factor-2 (FGF-2). *J Thromb Haemost* 2008; **6**: 176-183 [PMID: 17949478 DOI: 10.1111/j.1538-7836.2007.02808.x]

35   **Witsch E**, Sela M, Yarden Y. Roles for growth factors in cancer progression. *Physiology* (Bethesda) 2010; **25**: 85-101 [PMID: 20430953 DOI: 10.1152/physiol.00045.2009]

36   **Shu YJ**, Weng H, Bao RF, Wu XS, Ding Q, Cao Y, Wang XA, Zhang F, Xiang SS, Li HF, Li ML, Mu JS, Wu WG, Liu YB. Clinical and prognostic significance of preoperative plasma hyperfibrinogenemia in gallbladder cancer patients following surgical resection: a retrospective and in vitro study. *BMC Cancer* 2014; **14**: 566 [PMID: 25096189 DOI: 10.1186/1471-2407-14-566]

37   **Palumbo JS**, Potter JM, Kaplan LS, Talmage K, Jackson DG, Degen JL. Spontaneous hematogenous and lymphatic metastasis, but not primary tumor growth or angiogenesis, is diminished in fibrinogen-deficient mice. *Cancer Res* 2002; **62**: 6966-6972 [PMID: 12460914]

38   **Zheng S**, Shen J, Jiao Y, Liu Y, Zhang C, Wei M, Hao S, Zeng X. Platelets and fibrinogen facilitate each other in protecting tumor cells from natural killer cytotoxicity. *Cancer Sci* 2009; **100**: 859-865 [PMID: 19302289 DOI: 10.1111/j.1349-7006.2009.01115.x]

39   **Steinbrecher KA**, Horowitz NA, Blevins EA, Barney KA, Shaw MA, Harmel-Laws E, Finkelman FD, Flick MJ, Pinkerton MD, Talmage KE, Kombrinck KW, Witte DP, Palumbo JS. Colitis-associated cancer is dependent on the interplay between the hemostatic and inflammatory systems and supported by integrin alpha(M)beta(2) engagement of fibrinogen. *Cancer Res* 2010; **70**: 2634-2643 [PMID: 20233870 DOI: 10.1158/0008-5472.CAN-09-3465]

40   **Martino MM**, Briquez PS, Ranga A, Lutolf MP, Hubbell JA. Heparin-binding domain of fibrin(ogen) binds growth factors and promotes tissue repair when incorporated within a synthetic matrix. *Proc Natl Acad Sci USA* 2013; **110**: 4563-4568 [PMID: 23487783 DOI: 10.1073/pnas.1221602110]

41   **Staton CA**, Brown NJ, Lewis CE. The role of fibrinogen and related fragments in tumour angiogenesis and metastasis. *Expert Opin Biol Ther* 2003; **3**: 1105-1120 [PMID: 14519075 DOI: 10.1517/14712598.3.7.1105]

42   **Van Cutsem E**, Arends J. The causes and consequences of cancer-associated malnutrition. *Eur J Oncol Nurs* 2005; **9** Suppl 2: S51-S63 [PMID: 16437758 DOI: 10.1016/j.ejon.2005.09.007]

43   **Fleck A**, Raines G, Hawker F, Trotter J, Wallace PI, Ledingham IM, Calman KC. Increased vascular permeability: a major cause of hypoalbuminaemia in disease and injury. *Lancet* 1985; **1**: 781-784 [PMID: 2858667 DOI: 10.1016/S0140-6736(85)91447-3]

44   **Loeffler S**, Fayard B, Weis J, Weissenberger J. Interleukin-6 induces transcriptional activation of vascular endothelial growth

factor (VEGF) in astrocytes in vivo and regulates VEGF promoter activity in glioblastoma cells via direct interaction between STAT3 and Sp1. *Int J Cancer* 2005; **115**: 202-213 [PMID: 15688401 DOI: 10.1002/ijc.20871]

45   **Rothschild MA**, Oratz M, Schreiber SS. Serum albumin. *Hepatology* 1988; **8**: 385-401 [PMID: 3281888 DOI: 10.1002/hep.1840080234]

46   **Deehan DJ**, Heys SD, Simpson W, Herriot R, Broom J, Eremin O. Correlation of serum cytokine and acute phase reactant levels with alterations in weight and serum albumin in patients receiving immunotherapy with recombinant IL-2. *Clin Exp Immunol* 1994; **95**: 366-372 [PMID: 7511074 DOI: 10.1111/j.1365-2249.1994.tb07005.x]

47   **Nakashima J**, Tachibana M, Ueno M, Miyajima A, Baba S, Murai M. Association between tumor necrosis factor in serum and cachexia in patients with prostate cancer. *Clin Cancer Res* 1998; **4**: 1743-1748 [PMID: 9676850]

48   **Mantovani A**, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* 2008; **454**: 436-444 [PMID: 18650914 DOI: 10.1038/nature07205]

ORIGINAL ARTICLE

Observational Study

Fatigue is not associated with vitamin D deficiency in inflammatory bowel disease patients

Svein Oskar Frigstad, Marte Lie Høivik, Jørgen Jahnsen, Milada Cvancarova, Tore Grimstad, Ingrid Prytz Berset, Gert Huppertz-Hauss, Øistein Hovde, Tomm Bernklev, Bjørn Moum, Lars-Petter Jelsness-Jørgensen

Svein Oskar Frigstad, Department of Medicine, Vestre Viken Bærum Hospital, Gjettum 1346, Norway

Svein Oskar Frigstad, Department of Research, Østfold Hospital Kalnes, Grålum 1714, Norway

Marte Lie Høivik, Bjørn Moum, Department of Gastroenterology, Oslo University Hospital, Oslo 0424, Norway

Jørgen Jahnsen, Department of Gastroenterology, Akershus University Hospital, Lørenskog 1478, Norway

Milada Cvancarova, Faculty of Health Sciences, Oslo Metropolitan University, Oslo 0130, Norway

Tore Grimstad, Department of Gastroenterology, Stavanger University Hospital, Stavanger 4068, Norway

Ingrid Prytz Berset, Department of Medicine, Ålesund Hospital Trust, Ålesund 6026, Norway

Gert Huppertz-Hauss, Department of Gastroenterology, Telemark Hospital Trust, Skien 3710, Norway

Øistein Hovde, Department of Medicine, Innlandet Hospital Trust, Gjøvik 2819, Norway

Tomm Bernklev, Department of Research and Development, Vestfold Hospital Trust, Tønsberg 3103, Norway

Lars-Petter Jelsness-Jørgensen, Department of Gastroenterology, Østfold Hospital Kalnes, Grålum 1714, Norway

Lars-Petter Jelsness-Jørgensen, Department of Health Sciences, Østfold University College, Halden 1757, Norway

ORCID number: Svein Oskar Frigstad (0000-0001-7841-608X); Marte Lie Høivik (0000-0002-0104-465X); Jørgen Jahnsen (0000-0002-0419-2914); Milada Cvancarova (0000-0001-8947-8649); Tore Grimstad (0000-0001-6669-5843); Ingrid Prytz Berset (0000-0002-2014-8497); Gert Huppertz-Hauss (0000-0002-5693-8773); Øistein Hovde (0000-0003-4662-4153); Tomm Bernklev (0000-0002-9988-2271); Bjørn Moum (0000-0002-5884-4543); Lars-Petter Jelsness-Jørgensen (0000-0002-5465-1576).

Correspondence to: Svein Oskar Frigstad, MD, Doctor, Research Fellow, Department of Research, Østfold Hospital Kalnes, Postboks 300, Grålum 1714, Norway. s.o.frigstad@medisin.uio.no
Telephone: +47-91508600
Fax: +47-69863285

Abstract

AIM
To investigate if vitamin D deficiency is associated with fatigue in patients with inflammatory bowel disease (IBD).

METHODS
IBD patients were recruited from nine hospitals in the southeastern and western regions of Norway to participate in a multicenter cross-sectional study lasting from March 2013 to April 2014. Data were collected by interviews, from medical records and laboratory tests. The Fatigue Questionnaire (FQ) was used to measure fatigue. Linear and logistic regression models were applied to explore the possible association between vitamin D deficiency and total fatigue scores and chronic fatigue, respectively. The analyses were adjusted for age, gender, disease activity, depressive symptoms and sleep disturbance.

RESULTS
In total, 405 patients were included in the analyses, of which 227 (56%) had Crohn's disease (CD) and 178 (44%) had ulcerative colitis (UC). Vitamin D deficiency (< 50 nmol/L) was present in half (203/405) of the patients. Chronic fatigue was reported by 116 (29%) of all included patients with substantial fatigue reported by 194 (48%). Vitamin D levels were neither associated with total fatigue nor with chronic fatigue. Higher total fatigue scores and chronic fatigue were both associated with increased disease activity scores in patients with UC and CD, but not with increased CRP or fecal calprotectin. In UC patients, female gender was associated with fatigue in the univariate analysis, but no such difference was found when adjusted for elevated disease activity scores. Sleep disturbance and more depressive symptoms were associated with total fatigue scores in both UC and CD patients, but with chronic fatigue only in CD patients.

CONCLUSION
In this study, no significant association between fatigue and vitamin D deficiency in IBD patients was revealed.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Fatigue; Chronic fatigue; Vitamin D deficiency

Core tip: Fatigue is common in inflammatory bowel diseases (IBD) and is especially prevalent in active disease. We wanted to investigate if vitamin D deficiency was associated to fatigue in IBD as this is a common belief among both patients and physicians. To the best of our knowledge, no previous studies have investigated this possible association in IBD patients. We also wanted to take into account the multidimensional approach in understanding fatigue making it difficult to single out any one contributing factor. In this study, no significant association between fatigue and vitamin D deficiency in IBD patients was revealed.

INTRODUCTION

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are characterized by chronic, recurrent inflammation of the gastrointestinal tract[1-3]. IBD may present a variety of symptoms such as diarrhea with or without blood, abdominal pain, and fatigue. Patients with IBD often report the latter as one of their most troublesome symptoms[4].

Fatigue is common in IBD, with a prevalence of 44%-86% in active disease and 22%-41% in remission[5-7]. The patients usually report an overwhelming feeling of tiredness, reduced energy levels, reduced muscle strength, and cognitive impairment, which supports a multidimensional approach to understanding and measuring fatigue[5,6,8,9]. Chronic fatigue has been defined as severe fatigue persisting for at least six months leading to the substantial impairment of daily life[10,11].

Fatigue is associated with reduced health-related quality of life (HRQoL) and work ability[4,10,12-15]. In IBD, factors such as disease activity, disturbed sleep, anemia, pain and depression have all been associated with fatigue[5,6,10,16-19]. Anemia and pain are both related to more active disease, and nocturnal symptoms in IBD may be associated with sleep disturbance, a

known factor predisposing to fatigue. In addition, patients with severe fatigue are more likely to suffer from mood disorders such as depression, and there is an association between fatigue and psychological comorbidity[20].

Vitamin D deficiency has been reported to be associated with fatigue in cancer patients[21]. Moreover, vitamin D supplementation has been shown to be significantly associated with the improvement of fatigue symptoms in other chronic inflammatory conditions[22,23]. Several studies have shown that vitamin D deficiency is common in patients with IBD, that it is seen more often in patients with CD than in those with UC and that it is more frequent in these patients than in the general population[24-29].

Furthermore, vitamin D deficiency has been associated with disease activity in IBD patients[24,27,28,30-32]. Low vitamin D levels in IBD may result in increased fatigue that is partly due to more active disease. However, fatigue often persists after the patient has achieved clinical remission, without any objectively measured signs of intestinal inflammatory activity. This indicates that fatigue is not only a symptom related to inflammation[5].

The high prevalence of vitamin D deficiency may also be of potential importance in fatigue, as muscle weakness has been linked to vitamin D deficiency[33]. Skeletal muscle has vitamin D receptors and may require vitamin D for maximal function, and thus vitamin D deficiency may result in physical fatigue, one of the dimensions frequently measured in the assessment of fatigue[33].

The aim of this study was to investigate the possible association between vitamin D deficiency and fatigue in patients with IBD.

MATERIALS AND METHODS

Study area and population
Patients were recruited from nine hospitals in the southeastern and western part of Norway as part of an observational, multicenter cross-sectional study. Inclusion criteria were: Age ≥ 18 years, a verified diagnosis of IBD based on endoscopic, biochemical and histological findings according to the Lennard-Jones criteria[34], ability to read and understand Norwegian and to give written informed consent. The inclusion period lasted from March 2013 to April 2014. At each of the included centers, a senior gastroenterologist was responsible for the study.

Clinical, socio-demographic and laboratory variables
Data were collected by interviews, from medical records and laboratory tests. All data were collected at inclusion.

Serum 25-OH-D from all patients was analyzed by one central accredited laboratory with liquid chromatography tandem mass spectrometry (LC-MS/MS), and the method used for vitamin D analysis has

been compared to the LC-MS/MS method used in the vitamin D standardizing Program VDSP and showed good compliance[35]. Vitamin D deficiency was defined as 25-OH-D concentration < 50 nmol/L, and vitamin D insufficiency was defined as a 25-OH-D concentration of 50-75 nmol/L[33].

All other biochemical analyses were performed at the local laboratories at the participating centers. C-reactive protein (CRP) levels of 5 mg/L or higher were chosen to indicate active inflammation[36,37].

The stool samples for the measurement of fecal calprotectin were sent by mail and analyzed with Calpro Easy Extract (Calpro AS, Norway). Fecal calprotectin < 100 mg/kg was set as the cut-off for disease in remission, while higher levels were defined as active inflammation[38].

Clinical disease activity was measured with the Simple Clinical Colitis Activity Index (SCCAI) for UC and the Harvey Bradshaw Index (HBI) for CD[39,40]. For UC, an SCCAI score ≥ 5 was defined as active disease[36,37,41,42], and in CD, an HBI score ≥ 5 was defined as active disease[39,43].

Assessment of fatigue, depressive symptoms and quality of sleep
Fatigue was measured with the Fatigue Questionnaire (FQ)[8,44]. The FQ has been found to be suitable both for clinical and epidemiological purposes and has been translated into Norwegian and validated[8]. Two dimensions of fatigue are measured, physical fatigue (items 1-7) and mental fatigue (items 8-11). The sum of all items produces the total fatigue score. Each item score can be dichotomized (0 to 1 = 0, 2 to 3 = 1), giving a total score between 0 and 11. Chronic fatigue (CF) was defined as dichotomized FQ scores ≥ 4 with a duration > 6 mo, in accordance with previous studies[8,10,44,45].

The presence of depressive symptoms was measured with the Hospital Anxiety and Depression Scale (HADS), which has been translated into Norwegian and validated[46]. HADS is a 14-item questionnaire designed to screen for depression (7 items) and anxiety (7 items). Each item is scored from 0 to 3, and consequently the total score ranges from 0 to 21 for either depression or anxiety[46]. A higher score indicates an increased level of symptoms. In this study, depressive mood was defined as a HADS-D subscore ≥ 8, which is considered to be the most relevant score for the screening of depressive symptoms in chronic disease[47].

Sleep disturbance was measured with the first dimension of the Basic Nordic Sleep Questionnaire (BNSQ) on a 5-point Likert scale and dichotomized into normal sleep (scores 4 to 5) or sleep disturbance (scores 1 to 3)[48].

Statistical analysis
Normally distributed continuous variables were described with means and standard deviation (SD), and variables with skewed distributions were described with medians

and ranges. Crude differences between pairs of continuous variables were analyzed using Student's *t*-test when normally distributed, otherwise a non-parametric test (Kruskal Wallis test) was used. The crude association between pairs of categorical variables was analyzed with Chi-square test. To explore possible associations between the selected variables and fatigue, multiple regression models were fitted using fatigue as the dependent variable. For chronic fatigue, a logistic regression was fitted, and for total fatigue scores, a linear regression was used. Age and gender plus the variables that were statistically significant ($P < 0.1$) in univariate analyses were entered into the final multiple models. *P*-values < 0.05 were considered statistically significant in the multivariate analyses. As our analyses were considered as exploratory, no corrections for multiple testing were applied. All tests were two-sided. IBM SPSS Statistics for Windows version 24.0 (IBM Corp. Armonk, NY, United States, Released 2016) was used for all statistical analyses.

Ethical considerations
The Regional Committee for Medical and Health Research Ethics approved the study (2012/845/REK). All the study participants gave written, informed consent before inclusion in the study, and the study was performed in accordance with the Declaration of Helsinki.

RESULTS

In total, 452 patients were eligible, and their participation was requested; of these, 414 (92%) gave written consent. Nine of these 414 patients were excluded due to missing data, leaving 405 patients available for the analyses, of which 227 (56%) had CD and 178 (44%) had UC. There were no statistically significant differences between the UC and CD patients regarding age or gender, but the CD patients had significantly longer disease duration than the UC patients (median 11 *vs* 6 years, $P < 0.01$). Approximately half (203/405) of the patients had vitamin D deficiency[28]. For further details see Table 1.

The median score for total fatigue was similar in patients with UC and those with CD, see Table 1. There were no significant associations between vitamin D deficiency and mental, physical, and total fatigue scores. In addition, no significant differences in mean vitamin D levels between patients with and patients without chronic fatigue were found. Chronic fatigue was reported by 116 (29%) of all included patients with substantial fatigue (dichotomized FQ scores ≥ 4) reported by 194 (48%).

When separating patients into four groups according to (1) only chronic fatigue, (2) only depressive symptoms, (3) both chronic fatigue and depressive symptoms or (4) no chronic fatigue or depressive symptoms, there was no statistically significant association between the groups and their vitamin D levels, including when the data were adjusted for gender. No differences in total fatigue scores

or in the number of chronic fatigue cases were reported between patients receiving different medical treatments (data not shown).

In the multivariate linear regression analysis, when adjusted for age, gender, depressive symptoms, sleep disturbance and vitamin D level, higher total fatigue scores were associated with elevated disease activity scores in comparison to the total fatigue scores of patients in clinical remission in both UC and CD, but not with increased CRP or fecal calprotectin. In UC patients, female gender was associated with higher total fatigue scores in the univariate analysis, but this association disappeared in the multiple regression. Sleep disturbance and more depressive symptoms were associated with higher total fatigue in patients with UC and those with CD. The analyses are summarized in Tables 2 and 3.

In the multivariate logistic regression analysis, when adjusted for age, gender, depressive symptoms, sleep disturbance and vitamin D level, chronic fatigue was associated with elevated disease activity scores but not with increased CRP or fecal calprotectin, in contrast to patients in clinical remission regardless of diagnosis. In UC patients, female gender was associated with higher odds for chronic fatigue in the univariate analysis, but no such difference was found when adjusted for elevated disease activity scores. Self-reported sleep disturbance and depressive mood (HADS-D ≥ 8) were associated with higher odds for chronic fatigue in CD patients but not in UC patients in the multivariate analyses adjusted for disease activity and gender. The analyses are summarized in Tables 4 and 5.

DISCUSSION

In this cross-sectional study of IBD patients, neither total fatigue scores nor chronic fatigue were associated with vitamin D deficiency. Both higher total fatigue scores and chronic fatigue were associated with elevated disease activity scores, indicating clinically active disease in both UC and CD patients, but not with objective inflammatory markers. Moreover, higher total fatigue scores were associated with more depressive symptoms, as well as disturbed sleep in both disease groups.

Vitamin D deficiency was reported by half of the patients in this study, and as reported in a previous paper, this is more common than in the general population[28]. Furthermore, vitamin D deficiency was associated with higher disease activity scores and relapse rates in CD, as well as increased inflammatory markers in UC[28]. The prevalence of fatigue and mean total fatigue scores were similar to those reported in previous studies in IBD patients[5,7,10].

To the best of our knowledge, no previous studies have investigated if vitamin D deficiency is associated with fatigue in IBD patients. However, vitamin D deficiency has been linked to fatigue in cancer patients[21].

Table 1 Socio-demographic and clinical data *n* (%)

	UC (*n* = 178)	CD (*n* = 227)	*P* value
Age median, years (range)	40 (18-76)	40 (18-77)	0.92
Gender			
Female	87 (48)	112 (49)	0.77
Disease duration median, years (range)	6 (0-46)	11 (0-50)	< 0.01
Total fatigue, median (range)	16 (3-30)	15 (0-33)	0.55
Chronic fatigue	52 (29)	65 (28)	0.93
25-OH-D concentration nmol/L			
< 50	78 (44)	125 (55)	0.07
50-75	76 (43)	68 (30)	< 0.01
> 75	24 (13)	34 (15)	0.61
HADS-D ≥ 8	25 (14)	32 (14)	0.99
Sleep disturbance	44 (25)	36 (16)	0.03
Disease activity			
SCCAI ≥ 5	53 (29)		
HBI ≥ 5		103 (46)	
Fecal calprotectin ≥ 100 mg/kg (missing *n* = 82)	61 (34)	86 (38)	
CRP ≥ 5 mg/L (missing *n* = 10)	50 (28)	76 (33)	
UC extent, Montreal classification			
E1 - Proctitis	19 (11)		
E2 - Left-sided colitis	58 (32)		
E3 - Extensive colitis	101 (56)		
CD localization, Montreal classification			
L1 - Terminal ileum		74 (32)	
L2 - Colon		48 (21)	
L3 - Ileocolon		75 (33)	
L4 - Upper GI		31 (14)	
Current use of medication			
Biologics	52 (29)	113 (50)	< 0.01
5-ASA	137 (77)	23 (10)	< 0.01
Prednisolone	26 (14)	19 (8)	0.05
AZA / MTX	46 (26)	87 (38)	0.02

UC: Ulcerative colitis; CD: Crohn's disease; SCCAI: Simple Clinical Colitis Activity Index; HBI: Harvey Bradshaw index; ASA: Aminosalicylic acid; AZA: Azathioprine; MTX: Methotrexate; CRP: C-reactive protein; HADS-D: Hospital Anxiety and depression score - depression subscore.

Moreover, supplementation of vitamin D decreased fatigue scores in a Swedish study in patients with neurological disease[23]. In a study from the US of patients with different chronic conditions, not including IBD, vitamin D supplementation was also significantly associated with the improvement of fatigue symptoms[22]. These findings may suggest a possible role of vitamin D deficiency in the development of fatigue. As previously discussed, one explanation behind such an effect may be that insufficient vitamin D levels predispose patients to muscular weakness and physical fatigue. In the current study, however, no association between physical fatigue and vitamin D deficiency was found.

In addition, vitamin D has immunoregulatory proper-ties, which may attenuate intestinal inflammation[30,33,49]. As fatigue is more often reported in patients with active disease, one could speculate that vitamin D deficiency may contribute to disease activity, which in turn increases fatigue[5,10,16]. In the current study, however, vitamin D deficiency was not independently associated with fatigue. However, there was an association between fatigue and elevated disease activity scores in patients with UC and those with CD, but not with objective inflammatory markers such as CRP and fecal calprotectin. This suggests that factors other than inflammation may play an important role and that the total burden from

clinical disease activity may contribute relatively more to the experience of fatigue by the patient than just the intestinal inflammation.

Previous studies have also found pain and disturbed sleep to be of importance for the perception of fatigue in IBD[17,50]. This is in accordance with our results, where symptoms of disturbed sleep were consistently associated with both total fatigue scores and chronic fatigue for both UC and CD patients. Sleep disturbance has been shown to be highly prevalent in inflammatory conditions including IBD and has also been associated with higher fatigue scores[17,50]. Furthermore, there is evidence that sleep deprivation may increase inflammatory activity, but this has not been studied in IBD patients[50].

The finding that several symptoms not directly related to intestinal inflammation may influence fatigue is supported by a Swedish study on fatigue in gastrointestinal disorders that reported more severe fatigue in patients with functional gastrointestinal disorders than in those with organic gastrointestinal disorders[18]. Another Swedish study in irritable bowel syndrome has shown that patients with more severe symptoms report higher fatigue severity, supporting the importance of symptom burden in fatigue[51].

In contrast to previous studies, however, we found that clinical disease activity, depressive symptoms and

Table 2 Univariate and multivariate analysis of total fatigue and selected variables in ulcerative colitis (*n* = 178)

	Univariate B	95%CI	*P* value	Multivariate B	95%CI	*P* value
Age	-0.07	-0.13, -0.01	0.03	-0.05	-0.10, 0.00	0.04
Sex (ref. male)	2.30	0.80, 3.80	< 0.01	1.29	0.01, 2.57	0.05
25-OH-D < 50 nmol/L	Ref.					
25-OH-D 50-75	-0.27	-1.92, 1.39	0.75			
25-OH-D > 75	-0.93	-3.33, 1.47	0.45			
SCCAI ≥ 5	5.66	4.20, 7.12	< 0.01	4.25	2.75, 5.75	< 0.01
CRP ≥ 5 mg/L	0.35	-1.37, 2.07	0.69			
Calprotectin ≥ 100 mg/kg	0.61	-1.18, 2.40	0.50			
Depressive symptoms[1]	4.78	2.68, 6.88	< 0.01	2.37	0.44, 4.30	0.02
Sleep disturbance	4.17	2.50, 5.84	< 0.01	2.07	0.47, 3.67	0.01

[1]HADS: Hospital Anxiety and depression score - depression subscore ≥ 8. UC: Ulcerative colitis; SCCAI: Simple Clinical Colitis Activity Index; CRP: C-reactive protein; B: Regression coefficient.

Table 3 Univariate and multivariate analysis of total fatigue and selected variables in Crohn's disease (*n* = 227)

	Univariate B	95%CI	*P* value	Multivariate B	95%CI	*P* value
Age	-0.01	-0.07, 0.05	0.75	0.00	-0.06, 0.05	0.84
Sex (ref. male)	1.30	-0.16, 2.75	0.08	0.63	-0.67, 1.93	0.34
25-OH-D < 50 nmol/L	Ref.					
25-OH-D 50-75	-1.04	-2.79, 0.63	0.22			
25-OH-D > 75	-0.66	-2.79, 1.48	0.55			
HBI ≥ 5	4.17	2.81, 5.54	< 0.01	3.26	1.92, 4.59	< 0.01
CRP ≥ 5 mg/L	0.68	-0.89, 2.25	0.40			
Calprotectin ≥ 100 mg/kg	0.00	1.61, 1.61	1.00			
Depressive symptoms[1]	5.99	4.02, 7.94	< 0.01	4.72	2.82, 6.63	< 0.01
Sleep disturbance	4.04	2.10, 5.99	< 0.01	2.21	0.40, 4.03	0.02

[1]HADS-D: Hospital Anxiety and depression score - depression subscore ≥ 8. CD: Crohn's disease; SCCAI: Simple Clinical Colitis Activity Index; CRP: C-reactive protein; B: Regression coefficient.

sleep disturbance are independently associated with fatigue. Depressive symptoms have consistently been associated with fatigue, mainly due to an overlap of symptoms between these conditions. We believe that these are separate conditions, even if fatigue has been shown to predict the onset of depression[52]. Similar to our study, fatigue is often seen in patients with no history or current signs of psychological comorbidity[53]. In a Canadian study, perceived psychological stress was associated with the presence of fatigue, but depressive symptoms were not measured[17].

Previous studies in IBD patients have shown that fatigue is more commonly reported in female patients[6,7]. A similar observation has been made in a Norwegian study on fatigue in the general population[8]. In other studies, however, gender has not been shown to have a significant impact on fatigue[10,19]. The latter is in accordance with the current study, where female gender was not associated with fatigue when adjusted for disease activity.

Even with a cross-sectional design of our study, the number of patients included is relatively high, and the patients were recruited from several hospitals. The patients included can therefore be assumed to be representative of IBD patients treated in specialist care settings. The sample size and completeness of data, with few missing data from questionnaires, are important

strengths in this study. The choice of questionnaire used to measure fatigue may be of relevance when comparing results from different studies as this may influence both the number of cases and severity reported. We used the Fatigue Questionnaire because it has been validated in the general Norwegian population[8].

The duration of disease was longer in CD patients, and this may influence patient-reported outcomes such as fatigue, depressive symptoms and sleep disturbance. It is not known how disease duration influences these outcomes in chronic disease, but it may result in under-reporting, as patients may get used to fatigue symptoms over time. With self-reporting of symptoms, there is also a risk of recall bias.

Approximately 40% of the patients in the current study were treated with biologics. This may represent a selection bias of patients with more severe disease, with an expected higher prevalence of fatigue. On the other hand, effective medical treatment reducing the symptom burden may have improved fatigue scores in several patients. Only a few patients were treated on corticosteroids, not allowing for analyses on the effect use of steroids has on fatigue.

In conclusion, fatigue is common in IBD and is associated with clinical disease activity, depression and sleep disturbance. Our data did not reveal any association between vitamin D deficiency and fatigue, supporting

Table 4 Univariate and multivariate analysis of chronic fatigue in ulcerative colitis (*n* = 178)

	Univariate OR (95%CI)		*P* value	Multivariate OR (95%CI)		*P* value
Age, years	0.59	0.07, 1.02	0.59			
Gender (ref. male)	2.12	1.10, 4.11	0.025	1.80	0.90, 3.62	0.10
25-OH-D < 50 nmol/L (ref)	1.00					
25-OH-D 50-75	0.93	0.45, 1.84	0.80			
25-OH-D > 75	1.20	0.45, 3.18	0.72			
SCCAI ≥ 5	4.81	2.39, 9.67	< 0.01	4.47	2.20, 9.07	< 0.01
CRP ≥ 5 mg/L	1.20	0.57, 2.52	0.63			
Calprotectin ≥ 100 mg/kg	1.57	0.73, 3.39	0.25			
Depressive symptoms[1]	3.92	1.64, 9.36	< 0.01	2.55	0.98, 6.64	0.06
Sleep disturbance	2.71	1.33, 5.53	< 0.01	1.40	0.61, 3.19	0.43

[1]HADS-D: Hospital Anxiety and depression score - depression subscore ≥ 8. UC: Ulcerative colitis; SCCAI: Simple Clinical Colitis Activity Index; CRP: C-reactive protein.

Table 5 Univariate and multivariate analysis of chronic fatigue in Crohn's disease (*n* = 227)

	Univariate OR (95%CI)		*P* value	Multivariate OR (95%CI)		*P* value
Age, years	1.02	1.00, 1.04	0.10			
Gender (ref. male)	1.27	0.71, 2.26	0.41	1.11	0.61, 2.01	0.74
25-OH-D < 50 nmol/L (ref.)	1.00					
25-OH-D 50-75	0.65	0.33, 1.29	0.22			
25-OH-D > 75	1.25	0.56, 2.78				
HBI ≥ 5	2.71	1.50, 4.91	< 0.01	2.67	1.47, 4.87	< 0.01
CRP ≥ 5 mg/L	1.18	0.64, 2.19	0.59			
Calprotectin ≥ 100 mg/kg	0.56	0.29, 1.10	0.56			
Depressive symptoms[1]	4.77	2.19, 10.39	< 0.01	3.65	1.61, 8.27	< 0.01
Sleep disturbance	4.18	2.00, 8.74	< 0.01	3.02	1.38, 6.61	< 0.01

[1]HADS-D: Hospital Anxiety and depression score - depression subscore ≥ 8. CD: Crohn's disease; HBI: Harvey Bradshaw index; CRP: C-reactive protein.

a multidimensional approach in the understanding of fatigue. The possible associations we report need to be explored in further clinical studies before any certain conclusions can be drawn.

ARTICLE HIGHLIGHTS

Research background

Fatigue is common in inflammatory bowel diseases (IBD) and is especially prevalent in active disease. Also sleep disturbance, anemia, pain and depression all seem to influence fatigue. However, a relationship between vitamin D and fatigue has not been established.

Research motivation

We wanted to investigate if vitamin D deficiency was associated to fatigue in IBD as this is a common belief among both patients and physicians. To the best of our knowledge, no previous studies have investigated this possible association in IBD patients.

Research objectives

A relationship between vitamin D and fatigue has not been established. We wanted to explore this association and discover possible implications for our patients and further research.

Research methods

The research question was explored in a fairly large cohort of IBD patients from specialist care. The study was designed as an observational study, and all data were collected at inclusion. Linear and logistic regression models were applied to explore the possible association between vitamin D deficiency and total fatigue scores and chronic fatigue, respectively. All vitamin D analyses were done at the same laboratory.

Research results

In this study fatigue was commonly reported. Vitamin D levels were, however, neither associated with total fatigue nor with chronic fatigue. Higher total fatigue scores and chronic fatigue were both associated with increased disease activity scores, but not with objective markers of inflammation. Sleep disturbance and depressive symptoms were associated with total fatigue scores in both ulcerative colitis and Crohn's disease (CD) patients, but with chronic fatigue only in CD patients.

Research conclusions

In this study, fatigue was associated with clinical disease activity, depression and sleep disturbance. Our data did not reveal any association between vitamin D deficiency and fatigue, supporting a multidimensional approach in the understanding of fatigue.

Research perspectives

The possible associations we report need to be explored in further clinical studies. A randomized controlled trial with an interventional group receiving vitamin D supplementation may shed light on the possible benefits of vitamin D in patient reported outcomes in IBD.

ACKNOWLEDGEMENTS

REFERENCES

1   **Ponder A**, Long MD. A clinical review of recent findings in the epidemiology of inflammatory bowel disease. *Clin Epidemiol* 2013; **5**: 237-247 [PMID: 23922506 DOI: 10.2147/CLEP.S33961]

2   **Cosnes J**, Gower-Rousseau C, Seksik P, Cortot A. Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology* 2011; **140**: 1785-1794 [PMID: 21530745 DOI: 10.1053/j.gastro.2011.01.055]

3   **Ng SC**, Bernstein CN, Vatn MH, Lakatos PL, Loftus EV Jr, Tysk C, O'Morain C, Moum B, Colombel JF; Epidemiology and Natural History Task Force of the International Organization of Inflammatory Bowel Disease (IOIBD). Geographical variability and environmental risk factors in inflammatory bowel disease. *Gut* 2013; **62**: 630-649 [PMID: 23335431 DOI: 10.1136/gutjnl-2012-303661]

4   **Casellas F**, López-Vivancos J, Vergara M, Malagelada J. Impact of inflammatory bowel disease on health-related quality of life. *Dig Dis* 1999; **17**: 208-218 [PMID: 10754360 DOI: 10.1159/000016938]

5   **van Langenberg DR**, Gibson PR. Systematic review: fatigue in inflammatory bowel disease. *Aliment Pharmacol Ther* 2010; **32**: 131-143 [PMID: 20456309 DOI: 10.1111/j.1365-2036.2010.04347.x]

6   **Bager P**, Befrits R, Wikman O, Lindgren S, Moum B, Hjortswang H, Hjollund NH, Dahlerup JF. Fatigue in out-patients with inflammatory bowel disease is common and multifactorial. *Aliment Pharmacol Ther* 2012; **35**: 133-141 [PMID: 22059387 DOI: 10.1111/j.1365-2036.2011.04914.x]

7   **Huppertz-Hauss G**, Høivik ML, Jelsness-Jørgensen LP, Opheim R, Henriksen M, Høie O, Hovde Ø, Kempski-Monstad I, Solberg IC, Jahnsen J, Hoff G, Moum B, Bernklev T. Fatigue in a population-based cohort of patients with inflammatory bowel disease 20 years after diagnosis: The IBSEN study. *Scand J Gastroenterol* 2017; **52**: 351-358 [PMID: 27852169 DOI: 10.1080/00365521.2016.1256425]

8   **Loge JH**, Ekeberg O, Kaasa S. Fatigue in the general Norwegian population: normative data and associations. *J Psychosom Res* 1998; **45**: 53-65 [PMID: 9720855]

9   **Smets EM**, Garssen B, Bonke B, De Haes JC. The Multidimensional Fatigue Inventory (MFI) psychometric qualities of an instrument to assess fatigue. *J Psychosom Res* 1995; **39**: 315-325 [PMID: 7636775]

10   **Jelsness-Jørgensen LP**, Bernklev T, Henriksen M, Torp R, Moum BA. Chronic fatigue is more prevalent in patients with inflammatory bowel disease than in healthy controls. *Inflamm Bowel Dis* 2011; **17**: 1564-1572 [PMID: 21674713 DOI: 10.1002/ibd.21530]

11   **Swain MG**. Fatigue in chronic disease. *Clin Sci (Lond)* 2000; **99**: 1-8 [PMID: 10887052]

12   **Moum B**, Vatn MH, Ekbom A, Aadland E, Fausa O, Lygren I, Stray N, Sauar J, Schulz T. Incidence of Crohn's disease in four counties in southeastern Norway, 1990-93. A prospective population-based study. The Inflammatory Bowel South-Eastern Norway (IBSEN) Study Group of Gastroenterologists. *Scand J Gastroenterol* 1996; **31**: 355-361 [PMID: 8726303]

13   **Moum B**, Vatn MH, Ekbom A, Aadland E, Fausa O, Lygren I, Sauar J, Schulz T, Stray N. Incidence of ulcerative colitis and indeterminate colitis in four counties of southeastern Norway, 1990-93. A prospective population-based study. The Inflammatory Bowel South-Eastern Norway (IBSEN) Study Group of Gastroenterologists. *Scand J Gastroenterol* 1996; **31**: 362-366 [PMID: 8726304]

14   **Loftus EV Jr**. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. *Gastroenterology* 2004; **126**: 1504-1517 [PMID: 15168363]

15   **Bernklev T**, Jahnsen J, Henriksen M, Lygren I, Aadland E, Sauar J, Schulz T, Stray N, Vatn M, Moum B. Relationship between sick leave, unemployment, disability, and health-related quality of life in patients with inflammatory bowel disease. *Inflamm Bowel Dis* 2006; **12**: 402-412 [PMID: 16670530 DOI: 10.1097/01.MIB.0000218762.61217.4a]

16   **Czuber-Dochan W**, Ream E, Norton C. Review article: Description and management of fatigue in inflammatory bowel disease. *Aliment Pharmacol Ther* 2013; **37**: 505-516 [PMID: 23311461 DOI: 10.1111/apt.12205]

17   **Graff LA**, Vincent N, Walker JR, Clara I, Carr R, Ediger J, Miller N, Rogala L, Rawsthorne P, Lix L, Bernstein CN. A population-based study of fatigue and sleep difficulties in inflammatory bowel disease. *Inflamm Bowel Dis* 2011; **17**: 1882-1889 [PMID: 21830266 DOI: 10.1002/ibd.21580]

18   **Simrén M**, Svedlund J, Posserud I, Bjornsson ES, Abrahamsson H. Predictors of subjective fatigue in chronic gastrointestinal disease. *Aliment Pharmacol Ther* 2008; **28**: 638-647 [PMID: 18564325 DOI: 10.1111/j.1365-2036.2008.03770.x]

19   **van Langenberg DR**, Gibson PR. Factors associated with physical and cognitive fatigue in patients with Crohn's disease: a cross-sectional and longitudinal study. *Inflamm Bowel Dis* 2014; **20**: 115-125 [PMID: 24297056 DOI: 10.1097/01.MIB.0000437614.91258.70]

20   **Graff LA**, Clara I, Walker JR, Lix L, Carr R, Miller N, Rogala L, Bernstein CN. Changes in fatigue over 2 years are associated with activity of inflammatory bowel disease and psychological factors. *Clin Gastroenterol Hepatol* 2013; **11**: 1140-1146 [PMID: 23602816 DOI: 10.1016/j.cgh.2013.03.031]

21   **Dev R**, Del Fabbro E, Schwartz GG, Hui D, Palla SL, Gutierrez N, Bruera E. Preliminary report: vitamin D deficiency in advanced cancer patients with symptoms of fatigue or anorexia. *Oncologist* 2011; **16**: 1637-1641 [PMID: 21964001 DOI: 10.1634/theoncologist.2011-0151]

22   **Roy S**, Sherman A, Monari-Sparks MJ, Schweiker O, Hunter K. Correction of Low Vitamin D Improves Fatigue: Effect of Correction of Low Vitamin D in Fatigue Study (EViDiF Study). *N Am J Med Sci* 2014; **6**: 396-402 [PMID: 25210673 DOI: 10.4103/1947-2714.139291]

23   **Askmark H**, Haggård L, Nygren I, Punga AR. Vitamin D deficiency in patients with myasthenia gravis and improvement of fatigue after supplementation of vitamin D3: a pilot study. *Eur J Neurol* 2012; **19**: 1554-1560 [PMID: 22672742 DOI: 10.1111/j.1468-1331.2012.03773.x]

24   **Ulitsky A**, Ananthakrishnan AN, Naik A, Skaros S, Zadvornova Y, Binion DG, Issa M. Vitamin D deficiency in patients with inflammatory bowel disease: association with disease activity and quality of life. *JPEN J Parenter Enteral Nutr* 2011; **35**: 308-316 [PMID: 21527593 DOI: 10.1177/0148607110381267]

25   **Siffledeen JS**, Siminoski K, Steinhart H, Greenberg G, Fedorak RN. The frequency of vitamin D deficiency in adults with Crohn's disease. *Can J Gastroenterol* 2003; **17**: 473-478 [PMID: 12945007]

26   **Silvennoinen J**. Relationships between vitamin D, parathyroid hormone and bone mineral density in inflammatory bowel disease. *J Intern Med* 1996; **239**: 131-137 [PMID: 8568480]

27   **Sadeghian M**, Saneei P, Siassi F, Esmaillzadeh A. Vitamin D status in relation to Crohn's disease: Meta-analysis of observational studies. *Nutrition* 2016; **32**: 505-514 [PMID: 26837598 DOI: 10.1016/j.nut.2015.11.008]

28   **Frigstad SO**, Høivik M, Jahnsen J, Dahl SR, Cvancarova M, Grimstad T, Berset IP, Huppertz-Hauss G, Hovde Ø, Torp R, Bernklev T, Moum B, Jelsness-Jørgensen LP. Vitamin D deficiency in inflammatory bowel disease: prevalence and predictors in a Norwegian outpatient population. *Scand J Gastroenterol* 2017; **52**: 100-106 [PMID: 27603182 DOI: 10.1080/00365521.2016.1233577]

29   **Jahnsen J**, Falch JA, Mowinckel P, Aadland E. Vitamin D status, parathyroid hormone and bone mineral density in patients with inflammatory bowel disease. *Scand J Gastroenterol* 2002; **37**: 192-199 [PMID: 11843057]

30   **Jørgensen SP**, Hvas CL, Agnholt J, Christensen LA, Heickendorff L, Dahlerup JF. Active Crohn's disease is associated with low vitamin D levels. *J Crohns Colitis* 2013; **7**: e407-e413 [PMID: 23403039 DOI: 10.1016/j.crohns.2013.01.012]

31   **Blanck S**, Aberra F. Vitamin d deficiency is associated with ulcerative colitis disease activity. *Dig Dis Sci* 2013; **58**: 1698-1702 [PMID: 23334382 DOI: 10.1007/s10620-012-2531-7]

32   **Kabbani TA**, Koutroubakis IE, Schoen RE, Ramos-Rivers C, Shah N, Swoger J, Regueiro M, Barrie A, Schwartz M, Hashash JG, Baidoo L, Dunn MA, Binion DG. Association of Vitamin D Level With Clinical Status in Inflammatory Bowel Disease: A 5-Year Longitudinal Study. *Am J Gastroenterol* 2016; **111**: 712-719 [PMID: 26952579 DOI: 10.1038/ajg.2016.53]

33   **Holick MF**. Vitamin D deficiency. *N Engl J Med* 2007; **357**: 266-281 [PMID: 17634462 DOI: 10.1056/NEJMra070553]

34   **Lennard-Jones JE**. Classification of inflammatory bowel disease.

Scand J Gastroenterol Suppl 1989; **170**: 2-6; discussion 16-9 [PMID: 2617184]

35   **Cashman KD**, Dowling KG, Škrabáková Z, Kiely M, Lamberg-Allardt C, Durazo-Arvizu RA, Sempos CT, Koskinen S, Lundqvist A, Sundvall J, Linneberg A, Thuesen B, Husemoen LL, Meyer HE, Holvik K, Grønborg IM, Tetens I, Andersen R. Standardizing serum 25-hydroxyvitamin D data from four Nordic population samples using the Vitamin D Standardization Program protocols: Shedding new light on vitamin D status in Nordic individuals. *Scand J Clin Lab Invest* 2015; **75**: 549-561 [PMID: 26305421 DOI: 10.3109/003 65513.2015.1057898]

36   **Travis SP**, Higgins PD, Orchard T, Van Der Woude CJ, Panaccione R, Bitton A, O'Morain C, Panés J, Sturm A, Reinisch W, Kamm MA, D'Haens G. Review article: defining remission in ulcerative colitis. *Aliment Pharmacol Ther* 2011; **34**: 113-124 [PMID: 21615435 DOI: 10.1111/j.1365-2036.2011.04701.x]

37   **D'Haens G**, Sandborn WJ, Feagan BG, Geboes K, Hanauer SB, Irvine EJ, Lémann M, Marteau P, Rutgeerts P, Schölmerich J, Sutherland LR. A review of activity indices and efficacy end points for clinical trials of medical therapy in adults with ulcerative colitis. *Gastroenterology* 2007; **132**: 763-786 [PMID: 17258735 DOI: 10.1053/j.gastro.2006.12.038]

38   **Kristensen V**, Klepp P, Cvancarova M, Røseth A, Skar V, Moum B. Prediction of endoscopic disease activity in ulcerative colitis by two different assays for fecal calprotectin. *J Crohns Colitis* 2015; **9**: 164-169 [PMID: 25518057 DOI: 10.1093/ecco-jcc/jju015]

39   **Harvey RF**, Bradshaw JM. A simple index of Crohn's-disease activity. *Lancet* 1980; **1**: 514 [PMID: 6102236]

40   **Best WR**. Predicting the Crohn's disease activity index from the Harvey-Bradshaw Index. *Inflamm Bowel Dis* 2006; **12**: 304-310 [PMID: 16633052 DOI: 10.1097/01.MIB.0000215091.77492.2a]

41   **Walmsley RS**, Ayres RC, Pounder RE, Allan RN. A simple clinical colitis activity index. *Gut* 1998; **43**: 29-32 [PMID: 9771402]

42   **Gasche C**, Berstad A, Befrits R, Beglinger C, Dignass A, Erichsen K, Gomollon F, Hjortswang H, Koutroubakis I, Kulnigg S, Oldenburg B, Rampton D, Schroeder O, Stein J, Travis S, Van Assche G. Guidelines on the diagnosis and management of iron deficiency and anemia in inflammatory bowel diseases. *Inflamm Bowel Dis* 2007; **13**: 1545-1553 [PMID: 17985376 DOI: 10.1002/ibd.20285]

43   **Vermeire S**, Schreiber S, Sandborn WJ, Dubois C, Rutgeerts P. Correlation between the Crohn's disease activity and Harvey-Bradshaw indices in assessing Crohn's disease severity. *Clin Gastroenterol Hepatol* 2010; **8**: 357-363 [PMID: 20096379 DOI: 10.1016/j.cgh.2010.01.001]

44   **Chalder T**, Berelowitz G, Pawlikowska T, Watts L, Wessely S, Wright D, Wallace EP. Development of a fatigue scale. *J Psychosom Res* 1993; **37**: 147-153 [PMID: 8463991]

45   **Pawlikowska T**, Chalder T, Hirsch SR, Wallace P, Wright DJ, Wessely SC. Population based study of fatigue and psychological distress. *BMJ* 1994; **308**: 763-766 [PMID: 7908238]

46   **Zigmond AS**, Snaith RP. The hospital anxiety and depression scale. *Acta Psychiatr Scand* 1983; **67**: 361-370 [PMID: 6880820]

47   **Bjelland I**, Dahl AA, Haug TT, Neckelmann D. The validity of the Hospital Anxiety and Depression Scale. An updated literature review. *J Psychosom Res* 2002; **52**: 69-77 [PMID: 11832252]

48   **Partinen M**, Gislason T. Basic Nordic Sleep Questionnaire (BNSQ): a quantitated measure of subjective sleep complaints. *J Sleep Res* 1995; **4**: 150-155 [PMID: 10607192]

49   **Shivananda S**, Lennard-Jones J, Logan R, Fear N, Price A, Carpenter L, van Blankenstein M. Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European Collaborative Study on Inflammatory Bowel Disease (EC-IBD). *Gut* 1996; **39**: 690-697 [PMID: 9014768]

50   **Ranjbaran Z**, Keefer L, Farhadi A, Stepanski E, Sedghi S, Keshavarzian A. Impact of sleep disturbances in inflammatory bowel disease. *J Gastroenterol Hepatol* 2007; **22**: 1748-1753 [PMID: 17914945 DOI: 10.1111/j.1440-1746.2006.04820.x]

51   **Frändemark Å**, Jakobsson Ung E, Törnblom H, Simrén M, Jakobsson S. Fatigue: a distressing symptom for patients with irritable bowel syndrome. *Neurogastroenterol Motil* 2017; **29** [PMID: 27401139 DOI: 10.1111/nmo.12898]

52   **Dryman A**, Eaton WW. Affective symptoms associated with the onset of major depression in the community: findings from the US National Institute of Mental Health Epidemiologic Catchment Area Program. *Acta Psychiatr Scand* 1991; **84**: 1-5 [PMID: 1927557]

53   **van der Linden G**, Chalder T, Hickie I, Koschera A, Sham P, Wessely S. Fatigue and psychiatric disorder: different or the same? *Psychol Med* 1999; **29**: 863-868 [PMID: 10473313]

META-ANALYSIS

Fourth-generation quinolones in the treatment of *Helicobacter pylori* infection: A meta-analysis

Ying An, Ya Wang, Shuang Wu, You-Hua Wang, Xing Qian, Zhen Li, Ying-Jun Fu, Yong Xie

Ying An, Ya Wang, Shuang Wu, You-Hua Wang, Xing Qian, Yong Xie, Department of Gastroenterology, the First Affiliated Hospital of Nanchang University, Key Laboratory of Digestive Diseases of Jiangxi, Nanchang 330000, Jiangxi province, China

Ying An, Ya Wang, Ying-Jun Fu, School of Pharmacy, Nanchang University, Nanchang 330000, Jiangxi province, China

Zhen Li, Medical College, Nanchang University, Nanchang 330000, Jiangxi province, China

ORCID number: Ying An (0000-0003-2332-3825); Ya Wang (0000-0001-6644-2993); Shuang Wu (0000-0002-2849-6437); You-Hua Wang (0000-0002-0157-0721); Xing Qian (0000-0002-5209-0584); Zhen Li (0000-0002-2227-7612); Ying-Jun Fu (0000-0001-8884-7860); Yong Xie (0000-0002-5290-5579).

Abstract

AIM
To assess the efficacy and safety of fourth-generation quinolones for *Helicobacter pylori* (*H. pylori*) eradication, we conducted this systematic review and meta-analysis of randomized clinical trials.

METHODS
Major literature databases (PubMed, EMBASE and the Cochrane Central Register of Controlled Trials) were searched for relevant articles published prior to February 2018. We performed a meta-analysis of all randomized clinical trials that examined the efficacy of *H. pylori* eradication therapies and included fourth-generation quinolones in the experimental arm. Subgroup analyses by regions and different types of fourth-generation quinolones were also performed.

RESULTS
Ten studies including a total of 2198 patients were assessed. A meta-analysis of randomized controlled trials showed that the eradication rate of therapies containing non-fourth-generation quinolones was significantly lower

than that of therapies containing fourth-generation quinolones by intention-to-treat (ITT) analysis [75.4% *vs* 81.8%; odds ratio (OR) = 0.661; 95% confidence interval (CI): 0.447-0.977; *P* = 0.038]. This analysis also showed that the eradication rate of the therapies containing non-fourth-generation quinolones was inferior to that of therapies containing fourth-generation quinolones by per-protocol analysis (79.1% *vs* 84.7%; OR = 0.663; 95%CI: 0.433-1.016; *P* = 0.059). Moreover, the occurrence of side effects was significantly different between the control and experimental groups by ITT analysis (30.6% *vs* 19.5%; OR = 1.874; 95%CI: 1.120-3.137; *P* = 0.017). The sub-analyses also showed significant differences in moxifloxacin therapies *vs* other fourth-generation quinolone therapies (84.3% *vs* 71.9%) and in Asian *vs* European groups (76.7% *vs* 89.1%).

CONCLUSION
Therapies containing fourth-generation quinolones achieved a poor eradication rate in the treatment of *H. pylori* infection. Such regimens might be useful as a rescue treatment based on antimicrobial susceptibility testing. Different antibiotics should be chosen in different regions.

Key words: *Helicobacter pylori*; Fourth-generation quinolones; Eradication; Systematic review; Meta-analysis

Core tip: With the increase in the *Helicobacter pylori* (*H. pylori*) resistance rate, eradication is becoming increasingly challenging. This is the first meta-analysis comprehensively focused on fourth-generation quinolones for the treatment of *H. pylori* infection. Additionally, we found that fourth-generation quinolones had a higher eradication rate (81.8%) and a lower rate of incidence of side effects (19.5%). These findings will provide a specific basis for the clinical use of fourth-generation quinolones for *H. pylori* eradication.

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection plays a crucial role in the pathogenesis of gastrointestinal diseases, such as gastritis, non-ulcer dyspepsia, peptic ulcer diseases, and gastric cancer[1]. *H. pylori* infection affects approximately 50% of the population worldwide[2]. Its prevalence is approximately 70% in developing nations and approximately 20%-30% in developed nations[3]. Eradication of *H. pylori* facilitates peptic ulcer healing, reduces ulcer relapse rates, and prevents gastric

cancer[4]. In the past, the recommended treatment for eradicating *H. pylori* was 7 d of standard triple therapy (STT) consisting of a proton pump inhibitor (PPI) with clarithromycin (CAM) and amoxicillin (AMPC)[5]. However, with the wide use of the STT regimen, the eradication rate of *H. pylori* has declined to unacceptable levels over the last decade (< 80%) due to high resistance to metronidazole and clarithromycin[6]. A recent study on *H. pylori* resistance to antimicrobial agents reported that clarithromycin resistance has rapidly increased in many countries over the past decade, with resistance rates of approximately 18% in Europe, 30% in Japan, 40% in Turkey, and 50% in China; limited data are available for the United States[7-10]. The prevalence of *H. pylori* resistance to metronidazole is 33% in Europe and 40% in the United States, with a high resistance rate (50%-80%) in developing countries[10]. To overcome these difficulties, there is a need to evaluate novel regimens and antibiotics to identify effective alternative treatment strategies. Levofloxacin-based therapy is recommended by the Maastricht Ⅳ[11] and Maastricht Ⅴ Consensus Reports[12]. Nonetheless, according to studies, the resistance rate to levofloxacin is approximately 22.1% in Italy and 36.9% in China; a recent study surprisingly reported a resistance rate of 31.9% in the United States[13-15]. Jeong *et al*[16] reported that the eradication rate was 57.1% when levofloxacin was used. Fourth-generation quinolones, including moxifloxacin, sitafloxacin, gemifloxacin, and gatifloxacin, which have broad-spectrum antibacterial activity, are active against a variety of gram-negative and gram-positive bacteria[17]. Recent studies have shown that fourth-generation quinolones can increase drug penetration into bacterial cells, improve the strength of activity and have better bioavailability. This group of drugs inhibits the metabolism of bacterial cells by inhibiting DNA replication and therefore enhances antibacterial activity[18]. Furthermore, treatment with fourth-generation quinolones has achieved a high *H. pylori* eradication rate and has been recommended in some studies[19-22]. Nevertheless, Chung *et al*[23] reported that the eradication rate was not satisfactory when using fourth-generation quinolones.

To evaluate the efficacy and safety of therapies containing fourth-generation quinolones, we conducted a systematic review and meta-analysis of the available data. The primary outcome measures we assessed were eradication rates, side effects, and compliance of the therapies containing fourth-generation quinolones compared with those of therapies containing non-fourth-generation quinolones. Our outcomes will provide useful evidence for clinical practice[24].

MATERIALS AND METHODS

Literature sources
We searched the PubMed (to February 2018), EMBASE (to February 2018), and Cochrane Central Register of Controlled Trials (Issue 2, 2018) databases. The

following search terms were used for all databases: ("*Helicobacter pylori*" OR *H. pylori*) AND (Moxifloxacin OR Sitafloxacin OR Gemifloxacin OR Gatifloxacin); the search terms varied slightly among these databases. This meta-analysis was conducted according to the Preferred Reporting Item for Systematic Reviews and Meta-Analyses (PRISMA)[25].

Inclusion criteria
Articles eligible for inclusion in the meta-analysis met the following criteria: (1) randomized controlled trial (RCT) conducted; (2) methods used for the diagnosis of *H. pylori*, including the urea breath test (UBT), the rapid urease test (RUT), bacterial culture, histology, and/or fecal antigen test; (3) eradication rate made available; (4) eradication testing with UBT and/or histology performed at least 4 wk after the completion of therapy; and (5) eradication regimens in the experimental arm included fourth-generation quinolones.

Exclusion criteria
Studies were excluded under the following circumstances: (1) eradication data could not be confirmed; (2) articles and abstracts were written in a language other than English; (3) fourth-generation quinolones were included in two treatment arms; and (4) the experimental group and the control group included more than one variable (for example, the comparison of triple therapy and quadruple therapy; antibiotics and duration were different in both groups).

Data extraction
Three authors (An Y, Wang Y, and Wu S) independently extracted data from the selected studies. Any disagreements were resolved by consensus.

The extracted data included the following: the study design; number of enrolled patients in each treatment arm; diagnostic methods for confirming *H. pylori* infection before enrolling and re-checking strategies after completing the eradication study; publication time; name of the authors; location of the trial; drug regimens; duration of treatment; eradication rates by intention-to-treat (ITT) analysis and per-protocol (PP) analysis; number of successful and failed eradications; and percentage of adverse effects.

To avoid duplication of data, if a trial was repeatedly published by the same authors or institutions, only the most recently published or most informative study was included.

Risk of bias
The quality of RCTs with available full text was assessed using the risk of bias assessment tool developed by the Cochrane Handbook for Systematic Reviews of Interventions[26]. Two independent reviewers assessed the risk of bias through six domain-based evaluations, including selection bias (random sequence generation and allocation concealment), performance bias (blinding

of participants and personnel), detection bias (blinding of outcome assessment), attrition bias (incomplete outcome data), reporting bias (selective outcome reporting), and other bias. Each indicator was scored by low risk of bias, unclear risk of bias, and high risk of bias. Any disagreement was discussed and decided by a third reviewer. We also employed a funnel plot and Egger's test to assess the presence of publication bias.

Statistical analysis
The statistical analysis was performed using the meta-analysis software STATA12.0 (StataCorp LP, College Station, TX, United States). The primary outcomes of the meta-analysis were the *H. pylori* eradication rate and therapy-related side effects among the trials comparing the control and experimental groups based on ITT analysis. For each trial, we calculated the odds ratio (OR) for the primary measure. The ORs were presented with 95% confidence intervals (CIs); in addition, a *P*-value < 0.05 was considered significant. The degree of heterogeneity among the trial results was estimated using the χ^2 statistic (*P*-value < 0.10 considered significant) and the I^2 test (0%-25%, 25%-50%, 50%-75%, and > 75% represented insignificant, low, moderate, and high heterogeneity, respectively). If significant heterogeneity (*P* < 0.10 or I^2 > 50%) was achieved, we employed the random effects model to combine the effect sizes of the included studies. When no significant heterogeneity was found, we used fixed effects to pool the data. Additionally, subgroup analyses were performed based on the location and different types of fourth-generation quinolones.

RESULTS

Description of the studies
The bibliographical search yielded a total of 548 studies from PubMed, Embase, and the Cochrane Central Register of Controlled Trials. Among these articles, we excluded 144 due to duplication and 175 that were unrelated. We selected 229 potential studies for detailed assessment, among which 68 were excluded because there was no control group. We also excluded 67 review articles, comments, or letters. Thirty-three articles were excluded because of the inclusion of fourth-generation quinolones in two regimens, 14 articles were non-RCTs, 11 articles were excluded due to an inappropriate drug regimen, and 19 articles had data that could not be determined. Then, 17 articles were selected for further evaluation. Five articles were excluded because the data repeated those in other studies, one study did not state the methods for diagnosis of *H. pylori,* and one study was published in Japanese. Ultimately, 10 studies (two abstracts and eight full-text articles) met the inclusion criteria and were included in the systematic review and meta-analysis (Figure 1). These 10 studies[27-36] are summarized in Table 1 based on our meta-analysis. The quality assessment is reported in Table 2.

Figure 1 Flow diagram of the identified and selected trials.

Efficacy of H. pylori eradication

There were 10 studies with a total of 2198 patients in our meta-analysis; of these patients, 1107 received therapy without fourth-generation quinolone and 1091 received therapy with fourth-generation quinolone. The pooled eradication rates were 75.4% (835/1107) in the control group and 81.8% (892/1091) in the experimental group by ITT analysis. The pooled OR was 0.661 (95%CI: 0.447-0.977; P = 0.038) using the random effects model (I^2 = 66.2%, P = 0.000; Figure 2).

Moreover, the pooled eradication rates were 79.1% (835/1055) in the control group and 84.7% (892/1053) in the experimental group by PP analysis. The pooled OR was 0.663 (95%CI: 0.433-1.016; P = 0.059) using the random effects model (I^2 = 64.4%, P = 0.000).

The results of ITT showed that the eradication rates of therapies containing non-fourth-generation quinolones was significantly lower than those of therapies containing fourth-generation quinolones.

Subgroup analyses

Additional subgroup analyses for the meta-analysis were performed due to heterogeneity. We analyzed different types of fourth-generation quinolones, covering seven moxifloxacin trials and three other trials (including 1 sitafloxacin and 2 gemifloxacin). In the moxifloxacin subgroup, the pooled eradication rates were 77.3% (689/891) in the control group and 84.3% (733/870) in the experimental group (OR = 0.614, 95%CI: 0.403-0.935; P = 0.023; Figure 3) by ITT analysis, and the rates were 81.3% (689/847) in the control group and 87.3% (689/891) in the experimental group by

PP analysis (OR = 0.614, 95%CI: 0.395-0.956; P = 0.031). In the other subgroup, the pooled eradication rates were 67.6% (146/216) in the control group and 71.9% (159/221) in the experimental group (OR = 0.846, 95%CI: 0.274-2.614; P = 0.772; Figure 3) by ITT analysis, and the rates were 70.2% (146/208) in the control group and 74.6% (159/213) in the experimental group by PP analysis (OR = 0.860, 95%CI: 0.239-3.091; P = 0.817). This subgroup analysis showed that the regimen with moxifloxacin achieved a higher eradication rate than the regimen without moxifloxacin. However, there was no significant difference in the eradication rate in the other subgroup.

We also conducted subgroup analysis by region (seven trials in Asia and three trials in Europe). In the Asian subgroup, the pooled eradication rates of the control group and the experimental group were 76.5% (488/638) and 76.7% (493/643), respectively, by ITT analysis (OR = 1.051; 95%CI: 0.671-1.646; P = 0.827; Figure 4) and 79.6% (488/613) and 80.0% (493/616), respectively, by PP analysis (OR = 1.072; 95%CI: 0.627-1.833; P = 0.800). In the European subgroup, the pooled eradication rates of the control group and the experimental group were 74.0% (347/469) and 89.1% (399/448), respectively, by ITT analysis (OR = 0.661; 95%CI: 0.447-0.977; P = 0.000; Figure 4) and 78.5% (347/442) *vs* 91.3% (399/437), respectively, by PP analysis (OR = 0.361; 95% CI: 0.240-0.544; P = 0.000). The results showed that therapies containing fourth-generation quinolones may not be advisable treatments for *H. pylori* infection in Asia. However, the use of fourth-generation quinolones in Europe can

Table 1 Characteristics of studies included in the meta-analysis

Year-Author	Location	H. pylori infection initial diagnosis/re-checking	Control group-Day	Fourth-generation quinolone group-Day	Eradication rate (ITT) (control group/fourth-generation quinolone group)	Eradication rate (PP) (control group/fourth-generation quinolone group)	Compliance	Side effects
2017-Mansour Ghanaei, F	Iran	14C-UBT or histology	BPAC-10	BPAG-10	89% (81/91)/77% (70/91)	91% (81/90)/77.8% (70/90)		
2015-Masoodi, M	Iran	13C-UBT, RUT pathology test	OBAC-10	OBAG-10	61.6% (37/60)/66.6% (40/60)	67.2% (37/55)/72.7% (40/55)	97.1%/98.3%	37/19
2014-Rakici, H	Turkey	pistology, stool antigen test	LanAL-10	LanAM-10	89.4% (92/103)/87.8% (93/106)	92% (92/100)/91.8% (93/102)	96%/95.1%	-
2013-Murakami, K	Japan	culture method, RUT, UBT	LanAL-7	LanAS-7	43.1% (28/65)/70% (49/70)	43.7% (28/84)/72.1% (49/68)	98.4%/94.1%	11/11
2012-Zeng, Z	China	14C-UBT	EAC-7	EAM-7	78.9% (180/228)/79.4% (181/228)	82.9% (180/217)/84.2% (181/215)	-	-
2009-Lu, NH	China	14C-UBT	EAC-7	EAM-7	90.3% (28/31)/85.7% (24/28)	-	-	-
2008-Kilic, ZM	Turkey	gastroscopy, histology, RUT, 13C-UBT	RBCAC-14	RBCAM-14	76.7% (23/30)/66.7% (20/30)	76.7% (23/30)/66.7% (20/30)	100%/100%	11/13
			EAC-14	EAM-14	63.3% (19/30)/53.3% (16/30)	63.3% (19/30)/53.3% (16/30)	100%/100%	17/21
2007-Bago, P	Croatia	RUT, histology, culture test, 13C-UBT	LanMetC-7	LanMetM-7	70.4% (50/71)/93.5% (58/62)	75.8% (50/66)/96.7% (58/60)	-	
			LanAC-7	LanAM-7	78.2% (61/78)/86.4% (57/66)	80.2% (61/76)/90.5% 57/63)		
2005-Kist, M	Germany	13C-UBT	EAC-7	EAM-7	72.5% (58/80)/87.5% (70/80)	78% (58/74)/89% (70/79)	-	-
			ETC-7	ETM-7	75% (60/80)/90% (72/80)	79% (60/76)/92% (72/78)		
2005-Nista, EC	Italy	Histological examination, 13C-UBT	ETC-7	ETM-7	75% (60/80)/90% (72/80)	78.9% (60/76)/92.3% (72/78)		29/11
			EAC-7	EAM-7	72.5% (58/80)/87.5% (70/80)	78.4% (58/74)/88.6% (70/79)		26/10

A: Amoxicillin; B: Bismuth; C: Clarithromycin; E: Esomeprazole G: Gemifloxacin; Lan: Lansoprazole; L: Levofloxacin; M: Moxifloxacin; Met: Metronidazole; O: Omeprazole; P: Pantoprazole; S: Sitafloxacin; RBC: Ranitidine bismuth citrate; -: Not reported.

significantly improve the eradication rate.

Side effects
Of the 10 studies, four studies provided data regarding side effects. The results showed that common symptoms included nausea, diarrhea, black stool, and taste disturbance. The occurrence of total side effects in the control group was significantly higher than that in the experimental group by ITT analysis (30.6% *vs* 19.5%, OR = 1.874; 95%CI: 1.120-3.137; *P* = 0.017; Figure 5).

Compliance
Four studies included in the meta-analysis provided information about compliance. The results showed high compliance (> 95%), and there were no significant differences between the study groups.

Risk of bias in publication
Egger's regression test suggested that there was no significant bias (*P* = 0.725) in the ITT analysis, while the funnel plot showed a slightly asymmetrical distribution (Figure 6).

DISCUSSION

H. pylori infection is marked by a vast prevalence and strong association with various gastric diseases[37]. Therapeutic regimens range from STT to the present novel regimens,

Table 2 Risk assessment of included studies

Year-Author	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Other bias
2017-Mansour Ghanaei, F	L	H	H	L	L	L	L
2015-Masoodi, M	L	L	L	H	L	L	L
2014-Rakici, H	L	H	H	H	L	U	L
2013-Murakami, K	L	H	L	H	L	L	L
2012-Zeng, Z	U	U	U	U	L	U	U
2009-Lu, NH	U	U	U	U	L	U	U
2008-Kilic, ZM	L	H	H	H	L	L	U
2007-Bago, P	L	L	L	H	L	L	L
2005-Kist, M	H	H	H	H	L	H	L
2005- Nista, EC	L	H	H	H	L	L	L

L: Low risk of bias; H: High risk of bias; U: Unclear risk of bias.

Year_author		OR (95%CI)	Weight %
2017-Mansour-Ghanaei, F		2.43 (1.07, 5.51)	7.62
2015-Masoodi, M		0.80 (0.38, 1.70)	8.06
2014-Rakici, H		1.17 (0.50, 2.74)	7.40
2013-Murakami, K		0.32 (0.16, 0.66)	8.31
2012-Zeng, Z		0.97 (0.62, 1.53)	9.91
2009-Lu, N. H		1.56 (0.32, 7.65)	3.95
2008-Kilic, ZM		1.64 (0.53, 5.12)	5.82
2008-Kilic, ZM		1.51 (0.54, 4.24)	6.36
2007-Bago, P		0.16 (0.05, 0.51)	5.83
2007-Bago, P		0.57 (0.23, 1.37)	7.21
2005-Kist, M		0.38 (0.17, 0.86)	7.58
2005-Kist, M		0.33 (0.14, 0.81)	7.19
2005-Nista, EC		0.33 (0.14, 0.81)	7.19
2005-Nista, EC		0.38 (0.17, 0.86)	7.58
Overall (I^2 = 66.2%, P = 0.000)		0.66 (0.45, 0.98)	100.00

NOTE: Weights are from random effects analysis

0.0528   1   18.9

Figure 2 Forest plot of eradication rate of the therapies containing non-fourth-generation quinolones *vs* that of the therapies containing fourth-generation quinolones (intention-to-treat analysis).

such as quadruple therapy with bismuth, sequential treatment, concomitant therapy, and hybrid therapy[38,39]. However, the treatment effects are still not ideal due to

bacterial antibiotic resistance[40]. Thus, it is necessary to evaluate novel regimens or antibiotics[41]. With the resistance rate to the third-generation quinolone

Figure 3 Forest plot of the sub-analysis according to types of fourth-generation quinolone (intention-to-treat analysis).

levofloxacin continuing to increase, resulting in a low eradication rate[16], therapies containing fourth-generation quinolones might be suitable for the treatment of *H. pylori* infection.

This meta-analysis indicated that therapies containing fourth-generation quinolones had a higher clearance rate than other therapies by ITT and PP analyses. The mechanism of action of fourth-generation quinolones against *H. pylori* is to inhibit bacterial DNA gyrase, thus interfering with bacterial DNA replication[42]. These fourth-generation quinolones embed in the broken DNA chain and form complexes to inhibit nicking and closing activity, achieving a bactericidal effect[43]. However, according to Graham, who had given a report card to grade *H. pylori* therapy by ITT, the eradication rate is still poor (grade D, 81%-84%)[44]. This may be related to the low compliance of patients[27,33]. The choice of fourth-generation quinolones, the duration of treatment, and the difference in PPI also influenced the pooled eradication rates of therapies containing fourth-generation quinolones.
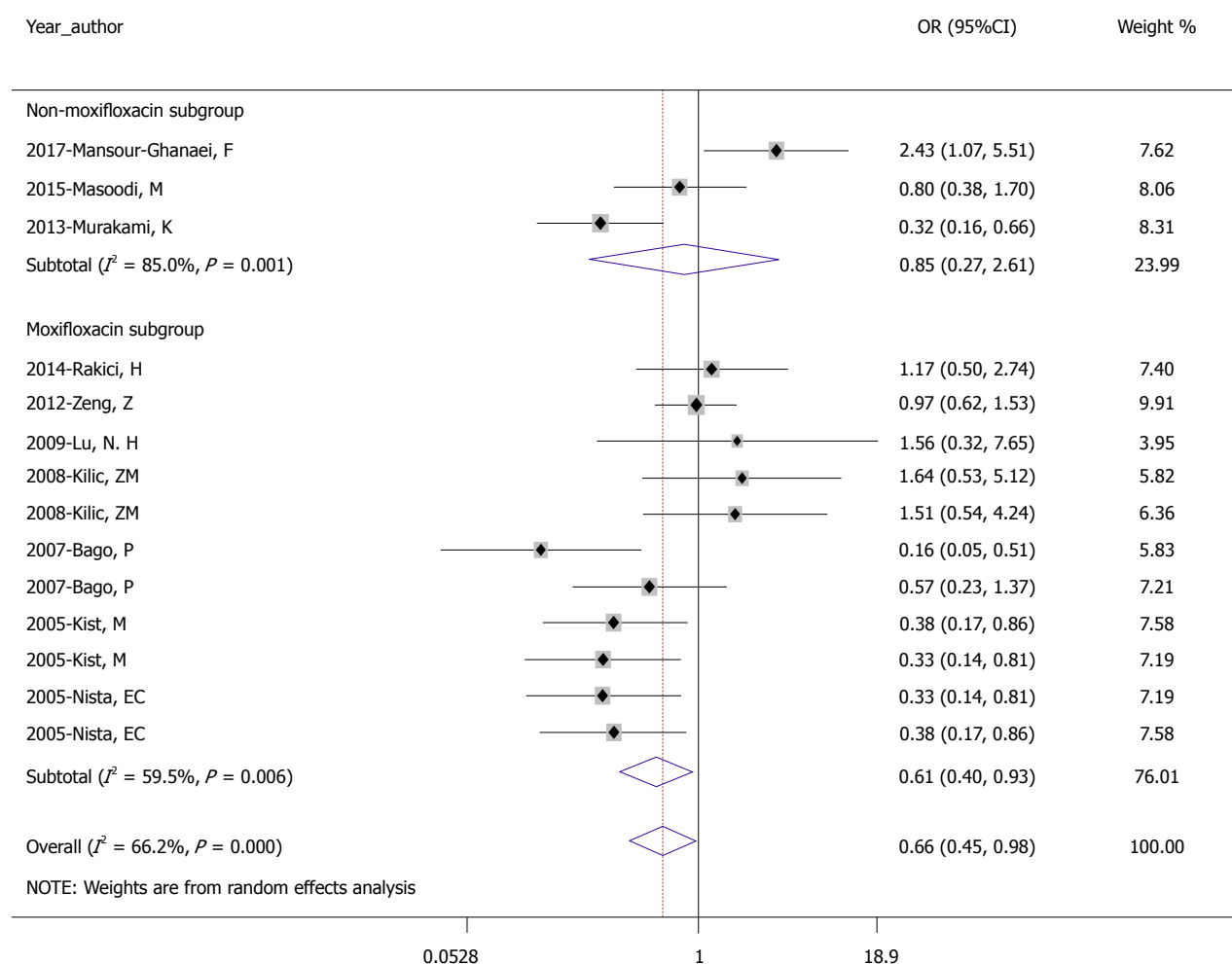
The subgroup analyses of antibiotic species conducted in this study demonstrated that regimens containing moxifloxacin were superior to those not containing moxifloxacin (84.3% *vs* 71.9%). This finding might be consistent with a previous systematic review[45], but the eradication rate was still less than 85% by ITT analysis. The main reason was that the resistance rate of *H. pylori* to moxifloxacin was higher, even reaching up to 27.0% when analyzed by the E-test[31]. This phenomenon reminds us that it is best to conduct a susceptibility test to choose antibiotics reasonably.

We also conducted subgroup analysis by region. The eradication rate of fourth-generation quinolone treatments in Europe was much higher than that in Asia (89.1% *vs* 76.7%). This difference may be due to the low utilization rate of antibiotics in Europe[8]. In Asia, the abuse of antibiotics is very common, which leads to a high drug resistance rate of *H. pylori*. According to a multiregion prospective 7-year study by Liu *et al*[46], the prevalence of *H. pylori* after moxifloxacin treatment was 17.2%. Resistance to moxifloxacin was reported to be similar to that of levofloxacin, ranging from 14.9% to 20.0% in Turkey[29]. The increasing antibiotic resistance rate makes the eradication of *H. pylori* more difficult.

The rate of incidence of adverse events in the control groups was higher than that in the experimental groups. The pooled OR (1.874) indicated that the use of fourth-generation quinolones in the treatment of *H. pylori* infection can reduce the incidence of adverse
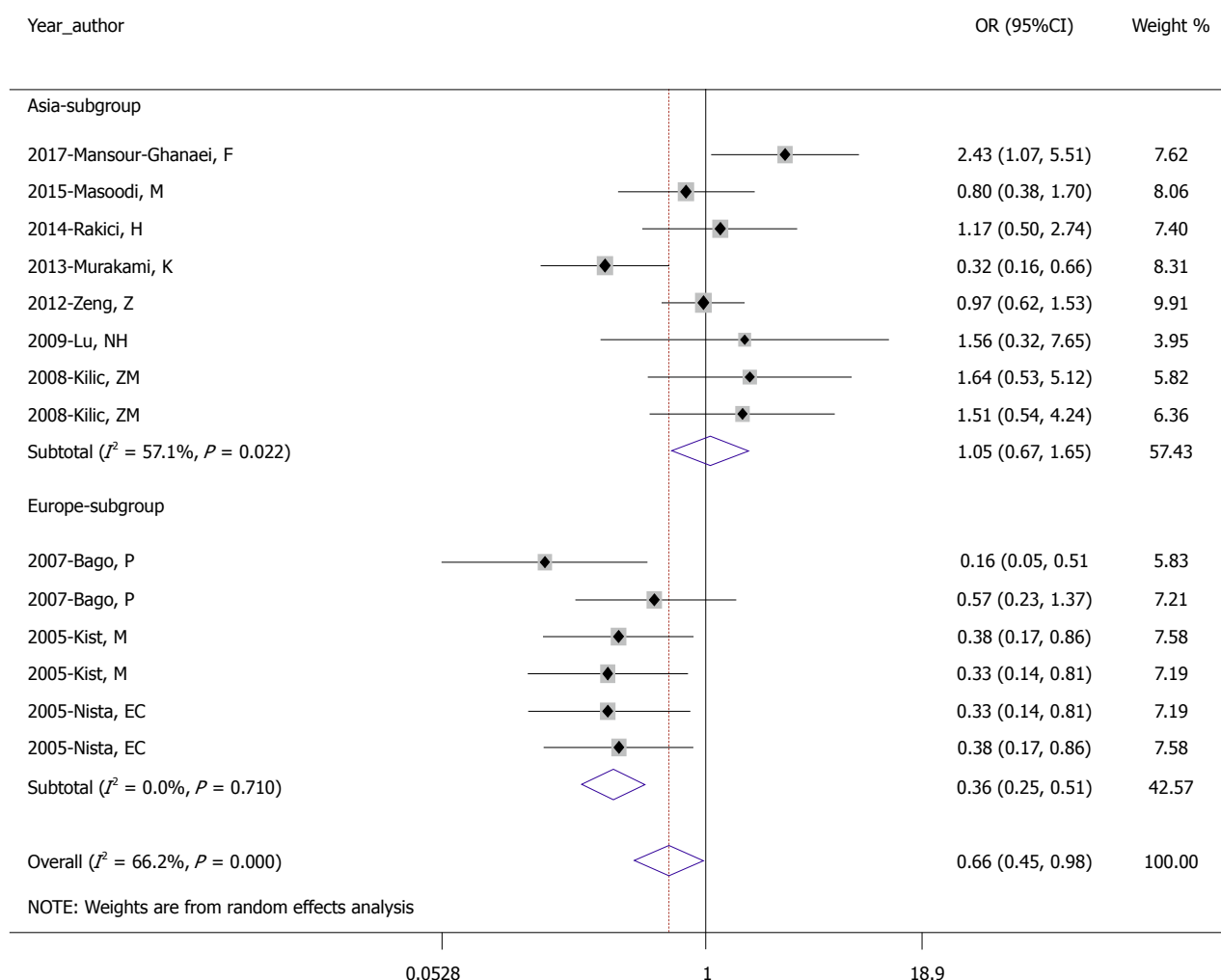
Figure 4 Forest plot of the sub-analysis according to region (intention-to-treat analysis).

reactions. This result indicates that therapies containing fourth-generation quinolones are safer.

The main limitation of this meta-analysis is potential biases. On the one hand, the largest number of studies was conducted using moxifloxacin; only one study used sitafloxacin, and two used gemifloxacin. This selection had a certain effect on the pooled eradication rate and may also be a particularly important issue in the use of a single antibiotic to eradicate *H. pylori* for clinical treatment. On the other hand, all included studies were performed in Europe and Asia, with no studies conducted in Africa or America. Because *H. pylori* infection occurs worldwide, our results may not be appropriate for global generalization. These two factors lead to the bias of conclusion. In addition, most of the studies in our meta-analysis had problems with concealment of allocation and blinding, which caused the selection bias. The restrictions on the language of publication also imply other bias, and thus our meta-analysis may not reflect all the outcomes.

Our analysis also implied other limitations. Most articles reporting a control arm were conducted using clarithromycin; our analysis is therefore especially lacking detailed data on levofloxacin. Two of the 10 included studies were abstracts, generating concerns regarding the data extraction and quality assessment of these studies and affecting the reliability of our results.

In conclusion, this meta-analysis indicates that therapies containing fourth-generation quinolones can achieve a higher eradication rate of *H. pylori* infection, but the eradication rate remains poor. In the absence of other drug options or in cases of patient allergy to penicillin, such regimens might be considered as a rescue treatment based on antimicrobial susceptibility testing. Further investigation is necessary to draw more solid conclusions about the use of fourth-generation quinolones in the treatment of *H. pylori* infection. In addition, we will study more effective therapies for *H. pylori* infection if necessary.

ARTICLE HIGHLIGHTS

Research background

The resistance of *Helicobacter pylori* (*H. pylori*) to antibiotics is increasing and often leads to the failure of eradication treatment. Recent studies have reported that therapies containing fourth-generation quinolones remain effective against antibiotic-resistant *H. pylori*. However, the efficacy and safety of these therapies require further study. This is the first meta-analysis comparing the curative
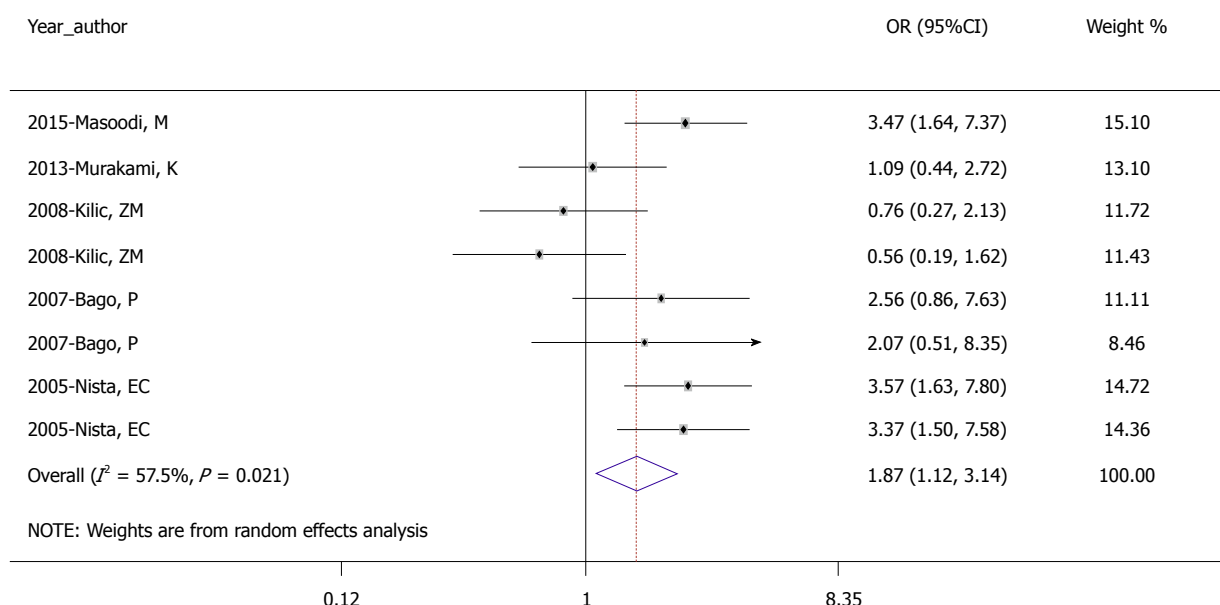
Year_author		OR (95%CI)	Weight %
2015-Masoodi, M		3.47 (1.64, 7.37)	15.10
2013-Murakami, K		1.09 (0.44, 2.72)	13.10
2008-Kilic, ZM		0.76 (0.27, 2.13)	11.72
2008-Kilic, ZM		0.56 (0.19, 1.62)	11.43
2007-Bago, P		2.56 (0.86, 7.63)	11.11
2007-Bago, P		2.07 (0.51, 8.35)	8.46
2005-Nista, EC		3.57 (1.63, 7.80)	14.72
2005-Nista, EC		3.37 (1.50, 7.58)	14.36
Overall (I^2 = 57.5%, P = 0.021)		1.87 (1.12, 3.14)	100.00

NOTE: Weights are from random effects analysis

0.12   1   8.35

Figure 5 Forest plot of the side effects of the therapies containing fourth-generation quinolones *vs* the therapies containing non-fourth-generation quinolones.
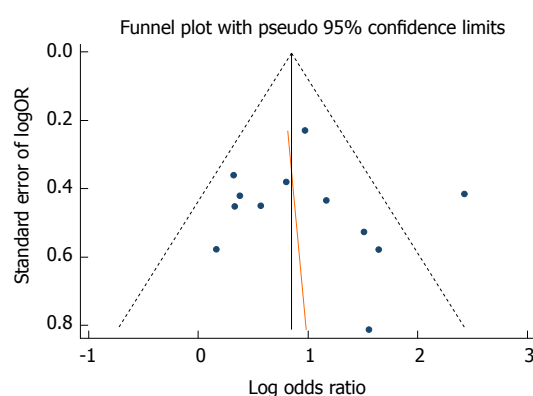
Figure 6 Funnel plot of the therapies containing non-fourth-generation quinolones *vs* the therapies containing fourth-generation quinolones (intention-to-treat analysis).

effect of fourth-generation quinolones with that of other therapies in regard to eradicating *H. pylori*.

Research motivation

In the Maastricht IV and Maastricht V Consensus Reports, levofloxacin-based therapy is recommended when the first treatment fails. Therapies containing fourth-generation quinolones are not mentioned. Our meta-analysis focused on eradication rates, side effects and compliance of therapies containing fourth-generation quinolones when compared with therapies using non-fourth-generation quinolones.

Research objectives

This meta-analysis aimed to clarify the effect of fourth-generation quinolones on the eradication of *H. pylori* infection and provide some evidence for clinical practice.

Research methods

The meta-analysis was conducted according to the PRISMA criteria. We searched the PubMed, EMBASE, and Cochrane Library databases. The outcome was to calculate the pooled eradication rate and therapy-related side effects among the

trials, comparing the control and experimental groups. We calculated the odds ratio of each trial for the primary measure. The odds ratios were presented with 95% confidence intervals, and a *P*-value < 0.05 was considered significant. This methodology was also performed for subgroup analysis.

Research results

Available data from 10 studies showed that treatment with a fourth-generation quinolone could achieve a higher *H. pylori* eradication rate and decrease the side effects, but the eradication rate is less than acceptable. Fourth-generation quinolones can significantly improve the eradication rate in Europe but not in Asia.

Research conclusions

Quinolone resistance increases with age and duration of use. It is essential for practitioners to use quinolone antibiotics in the clinic reasonably. This study comprehensively analyzed the role of fourth-generation quinolone in the treatment of *H. pylori* infection. Our results suggested that fourth-generation quinolones are not ideal for eradication of *H. pylori*. Treatment based on antibiotic susceptibility testing might be more valid and obtain a higher rate of eradication of *H. pylori* infection, particularly in areas where resistance to antibiotics develops rapidly.

Research perspectives

According to reports that mutations at positions 87 and 91 of gyrA are the main cause of *H. pylori* resistance to fourth-generation quinolones, we will continue to pay attention to the resistance rate to fourth-generation quinolones globally. We will also focus on rapid genotyping methods, such as detecting gyrA mutations in *H. pylori*. Further studies of sitafloxacin, gemifloxacin, and gatifloxacin are imperative to draw more solid conclusions about the use of fourth-generation quinolones for the eradication of *H. pylori* infection.

REFERENCES

1   **Liou JM**, Lin JT, Chang CY, Chen MJ, Cheng TY, Lee YC, Chen CC, Sheng WH, Wang HP, Wu MS. Levofloxacin-based and clarithromycin-based triple therapies as first-line and second-line treatments for Helicobacter pylori infection: a randomised comparative trial with crossover design. *Gut* 2010; **59**: 572-578 [PMID: 20427390 DOI: 10.1136/gut.2009.198309]

2   **Molina-Infante J,** Romano M, Fernandez-Bermejo M, Federico A, Gravina AG, Pozzati L, Garcia-Abadia E, Vinagre-Rodriguez G, Martinez-Alcala C, Hernandez-Alonso M , Miranda A,

Iovene MR, Pazos-Pacheco C, Gisbert JP. Optimized nonbismuth quadruple therapies cure most patients with Helicobacter pylori infection in populations with high rates of antibiotic resistance. Gastroenterology 2013; **145**: 121-128.e1 [PMID: 23562754 DOI: 10.1053/j.gastro.2013.03.050]

3   **Song M**, Ang TL. Second and third line treatment options for Helicobacter pylori eradication. *World J Gastroenterol* 2014; **20**: 1517-1528 [PMID: 24587627 DOI: 10.3748/wjg.v20.i6.1517]

4   **Wang B**, Lv ZF, Wang YH, Wang H, Liu XQ, Xie Y, Zhou XJ. Standard triple therapy for Helicobacter pylori infection in China: a meta-analysis. *World J Gastroenterol* 2014; **20**: 14973-14985 [PMID: 25356059 DOI: 10.3748/wjg.v20.i40.14973]

5   **Malfertheiner P**, Megraud F, O'Morain C, Bazzoli F, El-Omar E, Graham D, Hunt R, Rokkas T, Vakil N, Kuipers EJ. Current concepts in the management of Helicobacter pylori infection: the Maastricht III Consensus Report. *Gut* 2007; **56**: 772-781 [PMID: 17170018 DOI: 10.1056/NEJMcp1001110]

6   **Molina-Infante J**, Pazos-Pacheco C, Vinagre-Rodriguez G, Perez-Gallardo B, Dueñas-Sadornil C, Hernandez-Alonso M, Gonzalez-Garcia G, Mateos-Rodriguez JM, Fernandez-Bermejo M, Gisbert JP. Nonbismuth quadruple (concomitant) therapy: empirical and tailored efficacy versus standard triple therapy for clarithromycin-susceptible Helicobacter pylori and versus sequential therapy for clarithromycin-resistant strains. *Helicobacter* 2012; **17**: 269-276 [PMID: 22759326 DOI: 10.1111/j.1523-5378.2012.00947.x]

7   **Mitui M**, Patel A, Leos NK, Doern CD, Park JY. Novel Helicobacter pylori sequencing test identifies high rate of clarithromycin resistance. *J Pediatr Gastroenterol Nutr* 2014; **59**: 6-9 [PMID: 25222804 DOI: 10.1097/MPG.0000000000000380]

8   **Megraud F**, Coenen S, Versporten A, Kist M, Lopez-Brea M, Hirschl AM, Andersen LP, Goossens H, Glupczynski Y; Study Group participants. Helicobacter pylori resistance to antibiotics in Europe and its relationship to antibiotic consumption. *Gut* 2013; **62**: 34-42 [PMID: 22580412 DOI: 10.1136/gutjnl-2012-302254]

9   **Horiki N**, Omata F, Uemura M, Suzuki S, Ishii N, Iizuka Y, Fukuda K, Fujita Y, Katsurahara M, Ito T, Cesar GE, Imoto I, Takei Y. Annual change of primary resistance to clarithromycin among Helicobacter pylori isolates from 1996 through 2008 in Japan. *Helicobacter* 2009; **14**: 86-90 [PMID: 19751432 DOI: 10.1111/j.1523-5378.2009.00714.x]

10 **Thung I**, Aramin H, Vavinskaya V, Gupta S, Park JY, Crowe SE, Valasek MA. Review article: the global emergence of Helicobacter pylori antibiotic resistance. *Aliment Pharmacol Ther* 2016; **43**: 514-533 [PMID: 26694080 DOI: 10.1111/apt.13497]

11 **Malfertheiner P**, Megraud F, O'Morain CA, Atherton J, Axon AT, Bazzoli F, Gensini GF, Gisbert JP, Graham DY, Rokkas T, El-Omar EM, Kuipers EJ; European Helicobacter Study Group. Management of Helicobacter pylori infection--the Maastricht IV/ Florence Consensus Report. *Gut* 2012; **61**: 646-664 [PMID: 22491499 DOI: 10.1136/gutjnl-2012-302084]

12 **Malfertheiner P**, Megraud F, O'Morain CA, Gisbert JP, Kuipers EJ, Axon AT, Bazzoli F, Gasbarrini A, Atherton J, Graham DY, Hunt R, Moayyedi P, Rokkas T, Rugge M, Selgrad M, Suerbaum S, Sugano K, El-Omar EM; European Helicobacter and Microbiota Study Group and Consensus panel. Management of Helicobacter pylori infection-the Maastricht V/Florence Consensus Report. *Gut* 2017; **66**: 6-30 [PMID: 27707777 DOI: 10.1136/gutjnl-2016-312288]

13 **Saracino IM**, Zullo A, Holton J, Castelli V, Fiorini G, Zaccaro C, Ridola L, Ricci C, Gatta L, Vaira D. High prevalence of primary antibiotic resistance in Helicobacter pylori isolates in Italy. *J Gastrointestin Liver Dis* 2012; **21**: 363-365 [PMID: 23256118]

14 **Gao W**, Cheng H, Hu F, Li J, Wang L, Yang G, Xu L, Zheng X. The evolution of Helicobacter pylori antibiotics resistance over 10 years in Beijing, China. *Helicobacter* 2010; **15**: 460-466 [PMID: 21083752 DOI: 10.1111/j.1523-5378.2010.00788.x]

15 **Shiota S**, Reddy R, Alsarraj A, El-Serag HB, Graham DY. Antibiotic Resistance of Helicobacter pylori Among Male United States Veterans. *Clin Gastroenterol Hepatol* 2015; **13**: 1616-1624 [PMID: 25681693 DOI: 10.1016/j.cgh.2015.02.005]

16 **Jeong MH**, Chung JW, Lee SJ, Ha M, Jeong SH, Na S, Na BS,

Park SK, Kim YJ, Kwon KA, Ko KI, Jo Y, Hahm KB, Jung HY. [Comparison of rifabutin- and levofloxacin-based third-line rescue therapies for Helicobacter pylori]. *Korean J Gastroenterol* 2012; **59**: 401-406 [PMID: 22735872 DOI: 10.4166/kjg.2012.59.6.401]

17 **Mah FS**. Fourth-generation fluoroquinolones: new topical agents in the war on ocular bacterial infections. *Curr Opin Ophthalmol* 2004; **15**: 316-320 [PMID: 15232471 DOI: 10.1097/00055735-200408000-00007]

18 **Kłosińska-Szmurlo E**, Grudzień M, Betlejewska-Kielak K, Pluciński F, Biernacka J, Mazurek AP. Physicochemical properties of lomefloxacin, levofloxacin, and moxifloxacin relevant to the biopharmaceutics classification system. *Acta Chim Slov* 2014; **61**: 827-834 [PMID: 25551723]

19 **Hirata Y**, Serizawa T, Shichijo S, Suzuki N, Sakitani K, Hayakawa Y, Yamada A, Koike K. Efficacy of triple therapy with esomeprazole, amoxicillin, and sitafloxacin as a third-line Helicobacter pylori eradication regimen. *Int J Infect Dis* 2016; **51**: 66-69 [PMID: 27590563 DOI: 10.1016/j.ijid.2016.08.019]

20 **Mahmoudi L**, Farshad S, Seddigh M, Mahmoudi P, Ejtehadi F, Niknam R. High efficacy of gemifloxacin-containing therapy in Helicobacter Pylori eradication: A pilot empirical second-line rescue therapy. *Medicine* (Baltimore) 2016; **95**: e4410 [PMID: 27759625 DOI: 10.1097/MD.0000000000004410]

21 **Sugimoto M**, Sahara S, Ichikawa H, Kagami T, Uotani T, Furuta T. High Helicobacter pylori cure rate with sitafloxacin-based triple therapy. *Aliment Pharmacol Ther* 2015; **42**: 477-483 [PMID: 26075959 DOI: 10.1111/apt.13280]

22 **Gisbert JP**, Romano M, Molina-Infante J, Lucendo AJ, Medina E, Modolell I, Rodríguez-Tellez M, Gomez B, Barrio J, Perona M, Ortuño J, Ariño I, Domínguez-Muñoz JE, Perez-Aisa Á, Bermejo F, Domínguez JL, Almela P, Gomez-Camarero J, Millastre J, Martin-Noguerol E, Gravina AG, Martorano M, Miranda A, Federico A, Fernandez-Bermejo M, Angueira T, Ferrer-Barcelo L, Fernández N, Marín AC, McNicholl AG. Two-week, high-dose proton pump inhibitor, moxifloxacin triple Helicobacter pylori therapy after failure of standard triple or non-bismuth quadruple treatments. *Dig Liver Dis* 2015; **47**: 108-113 [PMID: 25454706 DOI: 10.1016/j.dld.2014.10.009]

23 **Chung KH**, Dong HL, Kim N, Shin CM, Jin HH, Sang HL, Lee D, Hong SO, Jin EH. Su1696 Efficacy of Second-Line Treatment for *Helicobacter pylori* Infection: Moxifloxacin-Containing Triple Therapy *vs*. Bismuth-Containing Quadruple Therapy. *Gastroenterology* 2012; **142**: S-483-S-484 [DOI: 10.1016/S0016-5085(12)61839-3]

24 **Moher D**, Liberati A, Tetzlaff J, Altman DG; PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med* 2009; **6**: e1000097 [PMID: 19621072 DOI: 10.1371/journal.pmed.1000097]

25 **Moher D**, Liberati A, Tetzlaff J, Altman DG; PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Int J Surg* 2010; **8**: 336-341 [PMID: 20171303 DOI: 10.1016/j.ijsu.2010.02.007]

26 **Higgins JP**, Green S. Cochrane Handbook for Systematic Reviews of Interventions Version 5.1.0 [updated March 2011]. The Cochrane Collaboration, 2011

27 **Mansour-Ghanaei F**, Pedarpour Z, Shafaghi A, Joukar F. Clarithromycin versus Gemifloxacin in Quadruple Therapeutic Regimens for *Helicobacter Pylori* Infection Eradication. *Middle East J Dig Dis* 2017; **9**: 100-106 [PMID: 28638586 DOI: 10.15171/mejdd.2017.58]

28 **Masoodi M**, Talebi-Taher M, Tabatabaie K, Khaleghi S, Faghihi AH, Agah S, Asadi R. Clarithromycin *vs*. Gemifloxacin in Quadruple Therapy Regimens for Empiric Primary Treatment of Helicobacter pylori Infection: A Randomized Clinical Trial. *Middle East J Dig Dis* 2015; **7**: 88-93 [PMID: 26106468]

29 **Rakici H**, Ayaz T, Akdogan RA, Bedir R. Comparison of levofloxacin- and moxifloxacin-based triple therapies with standard treatment in eradication of Helicobacter pylori as first-line therapy. *Digestion* 2014; **90**: 261-264 [PMID: 25547786 DOI: 10.1159/000369788]

30   **Murakami K**, Furuta T, Ando T, Nakajima T, Inui Y, Oshima T, Tomita T, Mabe K, Sasaki M, Suganuma T, Nomura H, Satoh K, Hori S, Inoue S, Tomokane T, Kudo M, Inaba T, Take S, Ohkusa T, Yamamoto S, Mizuno S, Kamoshida T, Amagai K, Iwamoto J, Miwa J, Kodama M, Okimoto T, Kato M, Asaka M; Japan GAST Study Group. Multi-center randomized controlled study to establish the standard third-line regimen for Helicobacter pylori eradication in Japan. *J Gastroenterol* 2013; **48**: 1128-1135 [PMID: 23307042 DOI: 10.1007/s00535-012-0731-8]

31   **Zeng Z**, Lv N, Hu F, Si J, Wu K, Jiang B, Liu W, Zhang J, Chen M, Hu P. Moxifloxacin-based triple therapy for Helicobacter pylori eradication: A multicenter randomized parallel-controlled study. *J Gastroenterol Hepatol* 2012; **27**: 3

32   **Lu NH**, Xie Y, Zhu X, Chen YX, Ma JH, He XX. Eradication therapy for Helicobacter pylori infection in patients with duodenal ulcers based on moxifloxacin triple therapy: a randomized controlled trial. *J Gastroenterol Hepatol* 2009; **24**: A15-A15

33   **Kiliç ZM**, Köksal AS, Cakal B, Nadir I, Ozin YO, Kuran S, Sahin B. Moxifloxacine plus amoxicillin and ranitidine bismuth citrate or esomeprazole triple therapies for Helicobacter pylori infection. *Dig Dis Sci* 2008; **53**: 3133-3137 [PMID: 18465244 DOI: 10.1007/s10620-008-0285-z]

34   **Bago P**, Vcev A, Tomic M, Rozankovic M, Marusić M, Bago J. High eradication rate of H. pylori with moxifloxacin-based treatment: a randomized controlled trial. *Wien Klin Wochenschr* 2007; **119**: 372-378 [PMID: 17634896 DOI: 10.1007/s00508-007-0807-2]

35   **Kist M**. How effective is moxifloxacin for the first-line treatment of patients with Helicobacter pylori infection? *Nat Clin Pract Gastroenterol Hepatol* 2005; **2**: 450-451 [PMID: 16224475 DOI: 10.1038/ncpgasthep0288]

36   **Nista EC**, Candelli M, Zocco MA, Cazzato IA, Cremonini F, Ojetti V, Santoro M, Finizio R, Pignataro G, Cammarota G, Gasbarrini G, Gasbarrini A. Moxifloxacin-based strategies for first-line treatment of Helicobacter pylori infection. *Aliment Pharmacol Ther* 2005; **21**: 1241-1247 [PMID: 15882245 DOI: 10.1111/j.1365-2036.2005.02412.x]

37   **Thamphiwatana S**, Gao W, Obonyo M, Zhang L. In vivo treatment of Helicobacter pylori infection with liposomal linolenic acid reduces colonization and ameliorates inflammation. *Proc Natl Acad Sci U S A* 2014; **111**: 17600-17605 [PMID: 25422427 DOI: 10.1073/pnas.1418230111]

38   **Apostolopoulos P**, Koumoutsos I, Ekmektzoglou K, Dogantzis P, Vlachou E, Kalantzis C, Tsibouris P, Alexandrakis G. Concomitant versus sequential therapy for the treatment of Helicobacter pylori infection: a Greek randomized prospective study. *Scand J Gastroenterol* 2016; **51**: 145-151 [PMID: 26435055 DOI: 10.3109/00365521.2015.1079646]

39   **Alhooei S**, Tirgar Fakheri H, Hosseini V, Maleki I, Taghvaei T, Valizadeh SM, Bari Z. A Comparison between Hybrid and Concomitant Regimens for *Helicobacter Pylori* Eradication: A Randomized Clinical Trial. *Middle East J Dig Dis* 2016; **8**: 219-225 [PMID: 27698972 DOI: 10.15171/mejdd.2016.24]

40   **Egan BJ**, Marzio L, O'Connor H, O'Morain C. Treatment of Helicobacter pylori infection. *Helicobacter* 2008; **13** Suppl 1: 35-40 [PMID: 18783520 DOI: 10.1111/j.1523-5378.2008.00639.x]

41   **Kwon YH**, Kim N, Lee JY, Choi YJ, Yoon K, Nam RH, Suh JH, Lee JW, Lee DH. Comparison of the efficacy of culture-based tailored therapy for Helicobacter pylori eradication with that of the traditional second-line rescue therapy in Korean patients: a prospective single tertiary center study. *Scand J Gastroenterol* 2016; **51**: 270-276 [PMID: 26452405 DOI: 10.3109/00365521.2015.1095352]

42   **Moore RA**, Beckthold B, Wong S, Kureishi A, Bryan LE. Nucleotide sequence of the gyrA gene and characterization of ciprofloxacin-resistant mutants of Helicobacter pylori. *Antimicrob Agents Chemother* 1995; **39**: 107-111 [PMID: 7695290 DOI: 10.1128/AAC.39.1.107]

43   **Lee JW**, Kim N, Nam RH, Park JH, Kim JM, Jung HC, Song IS. Mutations of Helicobacter pylori associated with fluoroquinolone resistance in Korea. *Helicobacter* 2011; **16**: 301-310 [PMID: 21762270 DOI: 10.1111/j.1523-5378.2011.00840.x]

44   **Graham DY**, Lee YC, Wu MS. Rational Helicobacter pylori therapy: evidence-based medicine rather than medicine-based evidence. *Clin Gastroenterol Hepatol* 2014; **12**: 177-186.e3; Discussion e12-e13 [PMID: 23751282 DOI: 10.1016/j.cgh.2013.05.028]

45   **Zhang G**, Zou J, Liu F, Bao Z, Dong F, Huang Y, Yin S. The efficacy of moxifloxacin-based triple therapy in treatment of Helicobacter pylori infection: a systematic review and meta-analysis of randomized clinical trials. *Braz J Med Biol Res* 2013; **46**: 607-613 [PMID: 23903685 DOI: 10.1590/1414-431X20132817]

46   **Liu DS**, Wang YH, Zeng ZR, Zhang ZY, Lu H, Xu JM, Du YQ, Li Y, Wang JB, Xu SP, Chen Y, Lan CH, Cheng H, Jiang MD, Zhang LX, Huo LJ, Chen SY, Zhang GX, Wu KC, Zhu X, Chen YX, Zhu Y, Shu X, Xie Y, Lu NH. Primary antibiotic resistance of Helicobacter pylori in Chinese patients: a multiregion prospective 7-year study. *Clin Microbiol Infect* 2018; **24**: 780.e5-780.e8 [PMID: 29138101 DOI: 10.1016/j.cmi.2017.11.010]

Baishideng®

World Journal of

Gastroenterology

World Journal of *Gastroenterology*

Contents

Contents

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Herwig Cerwenka, MD, Professor, Department of Surgery, Medical University of Graz, Graz A-8036, Austria

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 642 experts in gastroenterology and hepatology from 59 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

EDITORS FOR THIS ISSUE

MicroRNAs as non-invasive diagnostic biomarkers for gastric cancer: Current insights and future perspectives

Alexander Link, Juozas Kupcinskas

Alexander Link, Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University, Magdeburg 39120, Germany

Juozas Kupcinskas, Institute for Digestive Research and Department of Gastroenterology, Lithuanian University of Health Sciences, Kaunas LT-44307, Lithuania

ORCID number: Alexander Link (0000-0002-9514-4562); Juozas Kupcinskas (0000-0002-8760-7416).

Abstract

Non-invasive diagnostic biomarkers may contribute to an early identification of gastric cancer (GC) and improve the clinical management. Unfortunately, no sensitive and specific screening biomarkers are available yet and the currently available approaches are limited by the nature of the disease. GC is a heterogenic disease with various distinct genetic and epigenetic events that occur during the multifactorial cascade of carcinogenesis. MicroRNAs (miRNAs) are commonly deregulated in gastric mucosa during the *Helicobacter pylori* infection and in stepwise manner from chronic gastritis, through preneoplastic conditions such as atrophic gastritis and intestinal metaplasia, to early dysplasia and invasive cancer. Identification of miRNAs in blood in 2008 led to a great interest on miRNA-based diagnostic, prognostic biomarkers in GC. In this review, we provide the most recent systematic review on the existing studies related to miRNAs as diagnostic biomarkers for GC. Here, we systematically evaluate 75 studies related to differential expression of circulating miRNAs in GC patients and provide novel view on various heterogenic aspects of the existing data and summarize the methodological differences. Finally, we highlight several important aspects crucial to improve the future translational and clinical research in the field.

Key words: MicroRNA; Biomarkers; Screening; Stomach; Gastric cancer; Systematic review; Blood; Serum; Plasma

Core tip: Over the past years, large amount of data to microRNAs (miRNAs) in gastric cancer (GC) has been

published. We aimed to provide the critical, first of its kind in depth overview of existing studies to the miRNAs diagnostic biomarkers in GC. For this, we systematically reviewed published literature and identified 75 studies related specifically to microRNAs as blood-related non-invasive diagnostic biomarker in GC. This work provides a critical compendium to 106 studied microRNAs and summarizes the technical and methodological differences in reported studies. Furthermore, we highlight several aspects that need careful attention in future studies.

INTRODUCTION

Gastric cancer (GC) is a deadly disease with a great challenge in clinical management despite of a steady decline of the cancer incidence[1,2]. Despite of increasing understanding of genetic and epigenetic cancer events, the absence of non-invasive methods or biomarkers for early identification of GC is one of the biggest difficulties in GC. With an ongoing technical revolution, there is a great hope to find an appropriate way to solve this limitation. Non-coding RNAs (ncRNAs) and specifically microRNAs (miRNAs) have entered the "cancer-arena" for now more than 10 years ago[3] and much research has been done over the past decade. For several years we performed systematic analysis and reviewed the role of miRNAs in GC and potential of miRNAs as a biomarker in gastrointestinal cancers[4,5]. Since then, huge amount of data has been gained, making impossible to keep an overview of existing research. In this work, we performed a systematic search and reviewed published papers related to miRNAs as non-invasive biomarkers in GC. Because of the overwhelming amount of published data, we focused solely on miRNAs as non-invasive diagnostic biomarkers in GC and excluded the data to functional alterations, prognostic and predictive role. In the first part, we will briefly review the specific and unique issues related to GC crucial for understanding the disease, provide the compiling data showing the current stand of the research and highlight the need for the future development and new directions in the field.

GC - HETEROGENIC DISEASE

GC is a multifactorial heterogenic disease with unique cascade of genetic and epigenetic events leading to the cancer. There are multiple factors that, in more or less fashion, responsible for the clinical and biologically-relevant tumor heterogeneity, which may substantially impact an identification of potential diagnostic

biomarkers. Those factors include geographical differences in prevalence of the risk factors, genetic background of the population, environmental factors and probably nutrition. For instance, the prevalence of GC in Asian countries and Russia is higher than in United States, Canada and northern Europe. Interestingly, geographical differences correlate with anatomical localization of primary gastric tumors. Tumors in corpus-distal subtype are predominant in Asian countries and junctional-proximal subtype in Europe. Among the most important etiological factors that may influence the tumor biology at least during the process of carcinogenesis is the *Helicobacter pylori* (*H. pylori*) infection, which is now acknowledged as an infectious disease with all the consequences of prevention and treatment[6]. For in depth review of the role of *H. pylori* in GC development, we refer to several recent publications[7,8]. Briefly, *H. pylori* is a chronic infectious diseases that causes almost always an active chronic inflammation of the gastric mucosa[6]. The persistent chronic inflammation of gastric mucosa causes different range of molecular alterations with increasing loss and accumulation of changes that leads to the phase of atrophic gastritis (AG) with intestinal metaplasie (IM) and dysplasia, which may further progress to GC and is known as Correa's cascade[9]. AG and IM are well acknowledged preneoplastic stages of GC with an increased GC risk[10]. Close endoscopic follow up of patients with preneoplastic conditions and lesions is recommended[11]. The risk of *H. pylori*-related GC development may be associated with severity of mucosal inflammation and be partially dependent on bacterial virulence factors such as cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA)[12]. We and others have recently shown that VacA is probably one of the most important bacterial factors that correlate with mucosa inflammation[13] and is strongly associated with anti-CagA-IgG production[14]. *H. pylori* eradication is suggested as the most effective way in GC reduction in high-prevalence regions[15]; however, its value is beneficial mainly for primary and secondary GC prevention without strong diagnostic value in GC screening.

Historically, GC is divided based on the Lauren's classification into 2 histologic subtypes: intestinal and diffuse[16]. Lauren's classification is of the remarkable value in treatment decisions and has prognostic and predictive value. With an advance of high throughput technologies, the value of Lauren's classification has been questioned. The landmark work from The Cancer Genome Atlas (TCGA) has provided a new molecular classification subdividing GC in chromosomal instable (CIN), genomically stable (GS), microsatellite instable (MSI) and Epstein-Barr-Virus positive (EBV) tumor groups[17]. GS group shows relatively strong overlap with Lauren's diffuse type tumors. Hence, one of the main advantages of TCGA molecular classification may be in further subdivision of intestinal subtype of GC tumors in CIN, MSI and EBV. Those tumor subtypes carry not only unique molecular patterns relevant for understanding

the etiology but have potential predictive value for implementation of individualized novel therapeutic strategies.

CURRENTLY AVAILABLE BIOMARKERS FOR GC

To date, different molecules have been analyzed as potential biomarkers in patients with GC; however, as of 2018, there are no single blood-based biomarker that have sufficient sensitivity or specificity for implementation in GC screening routinely[8]. Several well-known antigens including carcinoembryonic antigen (CEA), cancer antigen 19-9 (CA19-9) and cancer antigen 72-4 (CA72-4) have been investigated in relation to GC[18]. Although the concentration of those antigens may be increased in some GC patients, the overall sensitivity of individual or combined CEA, CA19-9 and CA72-4 levels remains of insufficient discriminative power necessary for GC screening[18]. Besides typical "tumor-based" biomarkers, an effort has been made to establish a "functional" test for gastric mucosa also called "serological biopsy"[15]. Pepsinogen Ⅰ and Ⅱ (PGⅠ and PGⅡ) concentration correlate with AG, which is the preneoplastic condition of intestinal type of GC is intestinal type of GC, and is frequently used in Asian countries. In Europe the PGI and PGⅡ panels is expanded by the use of Gastrin-17 (G17). G17 is produced by the G-cells and stimulates the hydrochloric acid and pepsinogen production and therefore is part of the same physiological cascade as PGI. Unfortunately, the use of G17 is hampered by the stability of the peptide and the net benefit in addition to PGI and Ⅱ is a matter of ongoing research[19]. According to the recent systemic reviews and meta-analysis the sensitivity for AG identification is up to 70%[20,21]. Thus, PGI and PGⅡ may be helpful for serological identification of patients with probability of AG in regions with limited resources and availability of endoscopy. However, upper GI endoscopy with careful assessment of the gastric mucosa and targeted biopsies remains the gold-standard for GC screening and identification of patients at risk. One's upper GI endoscopy is performed, there is no additional diagnostic benefit of PGI/PGⅡ/G17 for instance in GC screening or risk assessment[22].

MiRNAs have gathered a lot of scientific attention during the past 10 years. As we have mentioned above, miRNAs are unique subgroup of ncRNAs with crucial role in multiple biological processes. MiRNAs are involved in regulation of different molecular pathways including cell differentiation, cell cycle progression or apoptosis through post-transcriptional regulation of gene expression[23]. Deregulation of miRNAs can influence carcinogenesis through mRNA targets encoding tumor suppressor genes or oncogenes[24]. Due to its unique biogenesis, miRNAs have several features that make them an attractive group of molecules in biomarker research field. MiRNAs are very stable and are easily and reproducibly retrieved from different biological material including tissues, blood, feces, saliva, ascites and even paraffin embedded blocks[25-32]. Due to those properties, miRNAs carry a huge potential as biomarkers and have been recently extensively explored in GC. For detailed information we kindly refer to our recent reviews[4,5].

MIRNA ALTERATIONS IN GC AND FUNCTIONAL ROLE

Multiple studies have demonstrated the differential expression of miRNAs in GC tissues and its functional role in GC has been suggested[4]. It is believed that miRNA alterations appear early in the cascade of the preneoplastic events. For instance, differential expression of miRNAs is identified in subjects with *H. pylori* infection and expression of miR-155 and miR-223 showed gradual increase in correlation to Correa's cascade both in antrum and corpus mucosa[25]. TCGA group has shown that different molecular subtypes of GC have unique miRNA expression profiles[17], which is in support of several other profiling studies[26,33]. Understanding of the mechanisms of miRNA expression such as CpG island promoter methylation, provides valuable information for biomarker research. For example, miR-137 was implicated in GI cancers showing differential methylation in colorectal cancer (CRC) and GC patients, although the magnitude of changes was superior in CRC[34]. MiR-29c expression is reduced early in gastric carcinogenesis and has been suggested as a diagnostic and therapeutic biomarker for patients with GC[35]. The interplay of the two transcription factors HNF4γ and NR2F2 and their coordinated regulation by miR-30 and miR-194, respectively, may represent a miRNA related network responsible for expression regulation of intestinal transcripts in the development of intestinal metaplasia[36].

Another growing field of miRNA research in GC is related to the analysis of single nucleotide polymorphisms (SNPs) in miRNA genes. Variation in miRNA-sequences may lead to the expression differences and modify regulatory function of miRNAs[37,38]. To date, both gene sequences encoding precursor miRNAs[39,40] as well as variations in miRNA binding regions of target genes[41] have been extensively explored in cancer studies. The most investigated SNPs in GC are related to miR-27a, miR-146a, miR-421, miR-449a; miR-196a-2, miR-492 and miR-608[42]. Although some associations between the risk of GC development and miRNA-related SNPs have been suggested, none of the identified associations is ready to be applied in clinical settings in particular in GC screening.

MIRNAS AS BIOMARKERS

Having shown the miRNA changes in tumor tissues, multiple research groups simultaneously evaluated the potential of miRNAs as non-invasive biomarkers in various specimens. Three publications related to large B-cell lymphoma, prostate and lung cancer appeared in 2008

strongly suggesting miRNAs as potential biomarkers for cancer[43–45]. This knowledge has been further extended to systematic analysis in feces in CRC[28], pancreatic cancer[29], and other diseases and specimens. Recently, it has been shown that miRNAs can be reproducibly measured in peritoneal fluid and ascites from cancer patients with peritoneal carcinomatosis and ascites[31,46]. It seems that basically any kind of body fluids (breast milk, urine, synovial fluids *etc*.) have measurable expression of miRNAs, which may potentially reflect a normal condition or be associated with pathophysiological alterations and therefore used as a biomarkers[5]. With existence of the overwhelming data to miRNAs in GC, we provide in the next chapter the most comprehensive summary of the exiting published data to miRNAs as non-invasive diagnostic biomarkers in GC.

METHODS AND LITERATURE SEARCH

To identify all available papers, we performed a systematic search with following steps: (1) Identify papers in MEDLINE/PUBMED using following criteria: gastric cancer, stomach cancer, microRNA, miRNA, biomarkers, plasma, serum, blood (until 30th November 2017); (2) We further screened all available abstracts manually one by one and excluded following papers: reviews, duplicates, retracted work, paper primarily related to miRNAs in tissues, *in vitro* or as prognostic or predictive biomarkers, papers without confirmed GC at the time point of the analysis or missing control group for direct comparison; (3) in January 2018 we updated the list including the papers published in December 2017 by applying more stringed criteria: diagnostic biomarkers, GC, plasma, serum. Overall, we obtained 75 original papers analyzing the expression of miRNAs in blood/serum/plasma between GC patients and controls. Among those, 18 papers refer to profiling of circulating miRNAs in GC compared to controls. Seventy-four full text papers and 1 abstract were systematically reviewed and the data were entered into the database. GraphPad Prism 7 (La Jolla, CA, United States) was used to create the figures.

MIRNA AS DIAGNOSTIC BIOMARKERS IN GC: TIMELINE AND PUBLICATION TRENDS

The first data to potential of miRNAs as diagnostic biomarkers in GC appeared in 2010 only two years after the first reports to detection of circulating miRNAs. We identified 75 publications from 2010 to 2017 where miRNA expression was studied in blood of GC patients and controls independently to the primary aim of the study. Over the period of 8 years, as shown in the Figure 1, we observed an increasing number of publication per year with maximum of 21 papers published in 2015 suggesting an increasing interest to the topic during the last years. To access the regional differences, we evaluated the origin of the used specimens as a surrogate. Majority of GC specimens and according published papers ori-

ginate from China (55/75 or 73.3%) followed by Japan, Taiwan, Korea and others, further suggesting the regional difference in priority of the research topic and potential clinical relevance (Figure 1B).

SCIENTIFIC EVIDENCE AND REPORTING QUALITY

As next, we aimed to systematically assess the reporting quality and translational potential of the published work. For this, we created 4 internal quality measures: (1) how many samples from GC patients was used; (2) is TNM-Stage reported; (3) is Lauren's classification reported; and (4) proportion of patients with TNM I / II in total amount of GC tumors. Figure 1C shows the number of GC samples used among the 75 published papers. The median number included in the study was 57 (range 3-285) and total 5699 specimens were analyzed. Among those, 5 reports included over 200 samples each with highest number of included samples published by Qiu *et al*[47].

As we have shown in the introduction part, the Lauren's classification is among the most valuable tools to assess the histological subtype, which further correlates with molecular subtype[17]. Unfortunately, the data to Lauren's classification were available only for 16% (12/74) of the studies. TNM staging was used as another reporting quality surrogate as it correlated with prognosis and potential biomarkers need to be able to identify early cancers. Interestingly, TNM staging was reported only in 69% (51/74) of studies, which may substantially limit the quality assessment of the published work (Figure 1D). Among the studies with reported TNM staging, the proportion of GC with relatively early stages (TNM I / II) was quite heterogenic between the studies (Figure 1E). Only two studies focused solely on samples from GC patients with TNM stage I and II, while 68.8% (35/51) of studies had more than 50% of samples from patients with metastatic GC (lymph node or distant metastases).

TECHNICAL DIFFERENCES AMONG THE STUDIES

Among the identified papers, 38 papers studied the expression of miRNAs in plasma, 32 studies in serum, 3 studies in blood and only 2 reports for peripheral blood mononuclear cell (PBMC) (Figure 2). For miRNA extraction, the mirVana, Trizol and miRNeasy were the most frequently used kits. There were substantial differences between the methods for detection/analysis. SYBR Green-based method was applied in 57% (42/74) more frequently used as TaqMan-based method (Figure 2C).

As next, we focused on the data to internal normalization of circulating miRNAs (Figure 2D). In several previous publications, RNU6b has been clearly criticized for unsuitability for normalization of blood samples as it shows different biogenesis, stability and may not reflect biogenesis of miRNAs[5]. Nevertheless, almost

Figure 1 Characteristics of the studies to microRNAs as diagnostic biomarkers in gastric cancer. In total we have identified 75 studies. A: Time trends in number of published papers starting 2010 to 2017. B: Number of papers dependent on the origin of GC tumor specimens studied. C: Total number of specimens per publication/analysis from GC patients studied. D: Number of papers reporting or not reporting TNM staging or Lauren´s classification. E: Proportion of patients with early (TNM stage Ⅰ and Ⅱ) to total number of GC specimen's studied. GC: Gastric cancer.

60% of studies used the RNU6b-method for internal normalization of blood specimens. In similar fashion, 15% of papers applied miR-16-based method (alone or in combination with other methods), although an increasing evidence suggests that miR-16-based method may not

be the best way for normalization of circulating miRNAs. Spiked-in-based method (most frequently cel-miR-39) is still considered as the most appropriate currently available methods for miRNAs normalization in blood and was used in up to 26% of studies, even though, it may

Figure 2 Characteristics of the studies to microRNAs as non-invasive diagnostic biomarkers in gastric cancer based on technical characteristics. A: Number of studies based on the used blood specimens. B: Number of papers using various extraction kits. C: Number of papers with various method for miRNA analysis. D: Number of studies using different normalization methods for miRNA analysis. Since several studies used one or several methods, the total number may exceed 75.

probably not be the perfect long-term solution for miRNA normalization.

Challenges with normalization of circulating miRNAs may probably be the most disappointing issue related to this topic. Several papers deal with the normalization issues and compare several methods, most frequently miR-16 and RNU6b. For instance, Song *et al*[48] studied the expression of several miRNAs in blood and showed that there is quite a large degree of variation among miRNAs. In particular, the expression of miR-21 was higher in stage IV GC patients; however, only if the samples were normalized to miR-16, miR-93 or miR-16 and miR-93 together. This was not the case if normalization was done based on the volume[48]. Interestingly, both miR-16 and miR-93 showed substantial variation and lower expression in healthy controls arguing against it usefulness as a normalizer. Peng *et al*[49] studied the expression of miR-191 and miR-425 in serum of 57

GC patients. No difference was found if the authors used RNU6b, while miR-16-based normalization led to significantly higher values of miR-191. In another study, Shiotani *et al*[50] also compared different normalization methods in GC samples from GC patients after endoscopic submucosal dissection. The authors conclude that only normalization to miR-16, but not RNU6b, led to the higher expression of miR-106b and let-7. According to our search (Table 1)[26,47,48,51-120], there are 3 reports to differential expression of miR-16 in GC with 399 studies samples[51-53]. Two reports show up- and one report downregulation of miR-16 in plasma and sera samples, further questioning the usefulness of miR-16 for normalization of circulating miRNAs.

The approach of miR-16-based normalization may be also called as a "proportional normalization" as rather the proportion between certain miRNAs (in comparison to miR-16) and not the absolute value of studied

Table 1 Differentially expressed circulating microRNAs in gastric cancer

microRNA	Changes in GC	Changes in GC tissue	Controls (*n*)	GC (*n*)	TNM reported	ROC	Validation	Source (Pl/Bl/Se)	qPCR	Normalization (qPCR)	Ref.
let-7a	↓	↓	30	69	Yes		Yes	Plasma	TaqMan	RNU6b	[56]
	↓	↓	45	80	Yes			Serum	SYBR	RNU6b	[57]
	↔		30	30	Yes			Plasma	TaqMan	RNU6b	[58]
let-7c	↔		202	214	No			Serum	SYBR	standard curve	[59]
let-7e	↑		82	82	No	0.7	Yes	Serum	TaqMan	cel-miR-39	[60]
let-7f	↔		30	30	Yes			Plasma	TaqMan	RNU6b	[58]
	↑		202	214	No			Serum	SYBR	standard curve	[59]
let-7g	↔		30	30	No			Serum	SYBR	miR-16	[61]
let-7i	↑		202	214	No			Serum	SYBR	standard curve	[59]
miR-1	↑		127	164	Yes			Serum	TaqMan	volume	[62]
	↔		30	30	No			Plasma	SYBR	cel-miR-39	[63]
miR-100	↑		47	50	Yes	0.71		Serum	TaqMan	RNU6b	[52]
miR-103	↑		14	17	No			Plasma	SYBR	Sp6	[64]
	↔		50	50	No	0.548		Plasma	SYBR	cel-miR-39	[65]
miR-106a	↑		30	69	Yes		Yes	Plasma	TaqMan	RNU6b	[56]
	↑		27	41	No	0.684		PBMC	SYBR	RNU6b	[66]
	↔		30	30	No			Plasma	SYBR	cel-miR-39	[63]
	↑	↑	22	48	NA			Serum	NA	NA	[67]
	↑	↑	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39 miR-191-5p	[68]
	↑		130	130	No	0.786	Yes	Serum+ Exosomes	TaqMan	cel-miR-39	[69]
miR-106b	↑	↑	30	69	Yes	0.721	Yes	Plasma	TaqMan	RNU6b	[56]
	↑		90	90	Yes	0.773	Yes	Plasma	SYBR	cel-miR-39	[63]
	↑		15	31	Yes			Serum	TaqMan	cel-miR-39	[70]
	↑	↑	20	20	Yes			Plasma	TaqMan	cel-miR-39 RNU6b	[71]
	↓		36	40	Yes	0.856		Serum	SYBR	QuantoEC	[72]
	↑		65	65	Yes	0.898		Plasma	TaqMan	RNU6b	[73]
miR-107	↑	↑	36	36	No	0.63		Serum	SYBR	5srRNA	[74]
	↑		14	14	Yes			Serum	TaqMan	RNU6b	[75]
	↔		50	50	No	0.563		Plasma	SYBR	cel-miR-39	[65]
miR-10b-5p	↑	↑	167	203	Yes	0.627	Yes	Serum+ Exosomes	SYBR	cel-miR-39 miR-16	[76]
miR-122	↔		36	96	Yes			Plasma	SYBR	ath-miR-159a	[77]
miR-1233	↓		3	3	No			Plasma	SYBR	RNU6b	[78]
miR-130a	↑	↑	41	41	No	0.905		Serum	SYBR	RNU6B	[79]
	↑	↑	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39 miR-191-5p	[68]
miR-132-3p	↑	↑	167	203	Yes	0.652	Yes	Serum+ Exosomes	SYBR	cel-miR-39 miR-16	[76]
miR-139	↓	↑	18	25	No	0.940		Plasma	SYBR	RNU6b	[80]
miR-140-5p	↑		50	50	Yes			Plasma	SYBR	RNU6B	[81]
miR-141	↓		3	3	No			Plasma	SYBR	RNU6b	[78]
	↓		14	17	No			Plasma	SYBR	Sp6	[64]
miR-142-3p	↓		285	285	Yes	0.839	Yes	Plasma	TaqMan	cel-miR-39	[47]
miR-143-3p	↔		73	206	Yes		Yes	Serum	SYBR	RNU6b	[82]
miR-144	↓	↓	40	96	Yes	0.821		Serum	SYBR	RNU6b	[83]
miR-146a	↔		15	31	Yes			Serum	TaqMan	cel-miR-39	[70]
	↔		73	206	Yes		Yes	Serum	SYBR	RNU6b	[82]
	↑	↔	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39 miR-191-5p	[68]
miR-146b	↑	↔	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39 miR-191-5p	[68]
miR-148a	↔		15	31	Yes			Serum	TaqMan	cel-miR-39	[70]
	↓		285	285	Yes	0.842	Yes	Plasma	TaqMan	cel-miR-39	[47]
	↓	↓	39	38	Yes	0.349		Plasma	TaqMan	miR-16-5p	[26]
miR-151-5p	↑		130	230	Yes	0.625	Yes	Plasma	TaqMan	RNU6b	[58]
miR-155	↑		20	30	No		Yes	Plasma	TaqMan	RNU6b	[84]
	↔		15	15	No			Plasma	TaqMan	RNU6b	[85]
miR-15b-5p	↑	↑	100	100	No			Plasma	SYBR	RNU6b	[86]
miR-16	↑		106	160	Yes	0.768-0.925	Yes	Plasma	TaqMan	cel-miR-39	[51]
	↑		47	50	Yes	0.90		Serum	TaqMan	RNU6b	[52]
	↓		129	189	Yes	0.772	Yes	Plasma	TaqMan	cel-miR-39 miR-16-5p	[53]

miRNA								Sample	Method	Normalizer	Ref
miR-17	↑		27	41	No	0.743		PBMC	SYBR	RNU6b	[66]
	↓		36	40	Yes	0.879		Serum	SYBR	QuantoEC	[72]
	↑		30	69	Yes		Yes	Plasma	TaqMan	RNU6b	[56]
	↔		30	30	No			Plasma	SYBR	cel-miR-39	[63]
	↔		20	20	No			Serum+ Exosomes	TaqMan	cel-miR-39	[69]
miR-181a	↑	↑	18	25	No	0.882		Plasma	SYBR	RNU6b	[80]
miR-181b	↓		89	92	Yes		Yes	Serum	SYBR	miR-16	[61]
miR-181c	↑	↑	60	30	No			Plasma	SYBR	RNU6b	[87]
miR-185	↑	↑	109	133	Yes	0.65	Yes	Plasma	SYBR	cel-miR-39 RNU6b	[88]
	↑	↔	167	203	Yes	0.637	Yes	Serum+ Exosomes	SYBR	cel-miR-39 miR-16	[76]
miR-187-3p	↑		61	61	Yes		Yes	Serum	SYBR	RNU6b	[89]
miR-18a	↑		50	50	Yes			Plasma	SYBR	RNU6B	[81]
	↑	↑	65	104	Yes	0.805		Plasma	TaqMan	RNU6b	[90]
miR-191	↑		82	82	No	0.63	Yes	Serum	TaqMan	cel-miR-39	[60]
	↑		58	57	Yes	0.849		Serum	TaqMan	miR-16	[49]
miR-192	↔		36	96	Yes			Plasma	SYBR	ath-miR-159a	[77]
miR-194	↑		3	3	No			Plasma	SYBR	RNU6b	[78]
	↔		50	50	No	0.512		Plasma	SYBR	cel-miR-39	[65]
miR-195	↓		285	285	Yes	0.765	Yes	Plasma	TaqMan	cel-miR-39	[47]
	↓	↓	36	62	Yes		Yes	Serum	SYBR	RNU6b	[91]
	↓		190	20	No			Plasma	(TaqMan)	global mean	[92]
	↑	↔	167	203	Yes	0.683	Yes	Serum+ Exosomes	SYBR	cel-miR-39 miR-16	[76]
mir-196a	↓	↑	14	17	No			Plasma	SYBR	Sp6	[64]
	↑	↑	126	98	Yes	0.864		Plasma	SYBR	miR-16	[93]
miR-196b	↑	↑	126	98	Yes	0.811		Plasma	SYBR	miR-16	[93]
miR-198	↔		30	30	Yes			Plasma	TaqMan	RNU6b	[58]
miR-199a-3p	↑		70	80	Yes	0.818	Yes	Plasma	TaqMan	RNU6b	[94]
	↑		130	230	Yes	0.837	Yes	Plasma	TaqMan	RNU6b	[58]
	↑		50	50	Yes			Plasma	SYBR	RNU6B	[81]
miR-19a	↑	↑	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39 miR-191-5p	[68]
miR-19b	↑	↔	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39 miR-191-5p	[68]
	↑		3	3	No			Plasma	SYBR	RNU6b	[78]
	↓		129	189	Yes	0.749	Yes	Plasma	TaqMan	cel-miR-39, miR-16-5p	[53]
	↑		130	130	No	0.769	Yes	Serum+ Exosomes	TaqMan	cel-miR-39	[69]
miR-200b	↓		14	17	no			Plasma	SYBR	Sp6	[64]
miR-200c	↑		15	52	Yes	0.715		Blood	SYBR	RNU6b 5SrRNA	[95]
	↑		100	98	Yes			Serum	SYBR	RNU6b	[96]
miR-203	↓		89	92	Yes		Yes	Serum	SYBR	miR-16	[61]
	↓		22	130	Yes	0.707	Yes	Serum	TaqMan	cel-miR-39	[97]
miR-204	↓		40	115	Yes		Yes	Serum	SYBR	RNU6b	[98]
miR-206	↓		150	150	Yes	0.89		Serum	TaqMan	cel-miR-39	[99]
miR-20a	↑		127	164	Yes			Serum	TaqMan	volume	[62]
	↑		90	90	Yes	0.859	Yes	Plasma	SYBR	cel-miR-39	[63]
	↑	↑	30	30	No			Plasma	SYBR	RNU6b	[100]
	↑	↑	109	133	Yes	0.67	Yes	Plasma	SYBR	cel-miR-39 RNU6b	[88]
	↑	↑	28	28	No			Plasma	SYBR	RNU6b	[101]
	↑	↔	167	203	Yes	0.637	Yes	Serum+ Exosomes	SYBR	cel-miR-39 miR-16	[76]
	↑		12	12	No			Serum	SYBR	cel-miR-39	[102]
miR-21	↑		30	69	Yes		Yes	Plasma	TaqMan	RNU6b	[56]
	↑		20	53	Yes	0.853		Blood	SYBR	RNU6b	[103]
	↑		70	70	Yes	0.794	Yes	Plasma	TaqMan	cel-miR-39	[104]
	↑		39	30	Yes	0.81		Serum	SYBR	miR-16	[105]
	↑		20	40	Yes			Serum	All-in-one	Volume miR-16, miR-93	[48]
	↔		90	90	Yes			Plasma	SYBR	cel-miR-39	[63]
	↔		15	31	Yes			Serum	TaqMan	cel-miR-39	[70]
	↔		15	15	No			Plasma	TaqMan	RNU6b	[85]
	↑		50	50	Yes	0.912		Serum	SYBR	RNU6b	[106]
	↑		50	50	Yes	0.898		PBMC	SYBR	RNU6b	[106]
	↑	↑	14	17	No			Plasma	SYBR	Sp6	[64]
	↑		89	92	Yes		Yes	Serum	SYBR	miR-16	[61]
	↑	↑	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39 miR-191-5p	[68]

miR-210	↑	↑	109	133	Yes	0.75	Yes	Plasma	SYBR	cel-miR-39 RNU6b	[88]
miR-212	↑	↔	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39; miR-191-5p	[68]
miR-218	↓		70	70	Yes	0.743	Yes	Plasma	TaqMan	cel-miR-39	[104]
	↓		56	68	Yes			Serum	SYBR	cel-miR-39	[107]
miR-220	↑	↔	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39; miR-191-5p	[68]
miR-221	↑		82	82	No	0.7	Yes	Serum	TaqMan	cel-miR-39	[60]
	↑		90	90	Yes	0.796	Yes	Plasma	SYBR	cel-miR-39	[63]
	↑		14	17	No			Plasma	SYBR	Sp6	[64]
miR-222	↑		82	82	No	0.65	Yes	Serum	TaqMan	cel-miR-39	[60]
	↑		56	114	Yes	0.85		Plasma	TaqMan	RNU6b	[108]
miR-223	↑		70	70	Yes	0.91	Yes	Plasma	TaqMan	cel-miR-39	[104]
	↑		15	31	Yes			Serum	TaqMan	cel-miR-39	[70]
	↑		47	50	Yes	0.85		Serum	TaqMan	RNU6b	[52]
	↑	↑	50	50	Yes	0.81		Plasma	SYBR	RNU6b	[109]
	↑		3	3	No			Plasma	SYBR	RNU6b	[78]
	↑	↑	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39; miR-191-5p	[68]
	↑	↑	20	15	Yes			Plasma	NCode	cel-miR-39/-54/-238; miR-16	[110]
	↑	↑	39	38	Yes	0.671		Plasma	TaqMan	miR-16-5p	[26]
miR-23a	↑		14	17	No			Plasma	SYBR	Sp6	[64]
miR-23b	↑		50	138	Yes	0.80		Plasma	SYBR	RNU6b	[111]
miR-25	↔		10	10	No			Plasma	TaqMan	cel-miR-39	[104]
	↑		106	160	Yes	0.694-0.925	Yes	Plasma	TaqMan	cel-miR-39	[51]
	↑	↑	20	20	Yes			Plasma	TaqMan	cel-miR-39; RNU6b	[71]
	↑	↑	109	133	Yes	0.65	Yes	Plasma	SYBR	cel-miR-39; RNU6b	[88]
	↔	↑	70	70	Yes			Plasma	TaqMan	RNU6B	[112]
	↑		14	14	Yes			Serum	TaqMan	RNU6b	[75]
	↑		65	65	Yes	0.817		Plasma	TaqMan	RNU6b	[73]
miR-26a	↓		285	285	Yes	0.882	Yes	Plasma	TaqMan	cel-miR-39	[47]
miR-26b	↔		30	30	Yes			Plasma	TaqMan	RNU6b	[58]
miR-27a	↑		127	164	Yes			Serum	TaqMan	volume	[62]
	↑		82	82	No	0.67	Yes	Serum	TaqMan	cel-miR-39	[60]
	↔		30	30	No			Plasma	SYBR	cel-miR-39	[63]
	↔		15	31	Yes			Serum	TaqMan	cel-miR-39	[70]
	↑	↑	35	35	Yes	0.70	Yes	Plasma	TaqMan	RNU6b	[85]
miR-27b	↑		82	82	No	0.66	Yes	Serum	TaqMan	cel-miR-39	[60]
miR-296	↑	↔	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39; miR-191-5p	[68]
	↑	↑	167	203	Yes	0.652	Yes	Serum+ Exosomes	SYBR	cel-miR-39 miR-16	[76]
miR-30a-5p	↔		20	20	No			Serum+ Exosomes	TaqMan	cel-miR-39	[69]
miR-30c	↑	↔	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39; miR-191-5p	[68]
miR-31	↓		89	92	Yes		Yes	Serum	SYBR	miR-16	[61]
miR-32	↑	↑	40	40	No			Plasma	SYBR	RNU6b	[113]
miR-323-3p	↑	↔	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39; miR-191-5p	[68]
miR-331	↑	↑	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39; miR-191-5p	[68]
miR-335	↓	↓	7	4	No			Plasma	TaqMan	RNU6b	[114]
miR-34	↔		30	30	No			Plasma	SYBR	cel-miR-39	[63]
miR-346	↓		14	17	No			Plasma	SYBR	Sp6	[64]
miR-34a	↑		127	164	Yes			Serum	TaqMan	volume	[62]
miR-365	↑	↔	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39; miR-191-5p	[68]
miR-370	↑	↑	12	40	Yes	0.79		Plasma	TaqMan	miR-16	[115]
miR-371-5p	↑		61	61	Yes		Yes	Serum	SYBR	RNU6b	[89]
miR-374	↑	↑	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39; miR-191-5p	[68]
miR-375	↓	↓	20	20	No	0.835		serum	TaqMan	RNU6b	[116]
	↓	↓	39	38	Yes	0.32		Plasma	TaqMan	miR-16-5p	[26]
miR-376a	↔	↑	108	65	Yes			Plasma	TaqMan	RNU6B	[117]
miR-376c	↑		82	82	No	0.71	Yes	Serum	TaqMan	cel-miR-39	[60]
	↔		30	30	No			Plasma	SYBR	cel-miR-39	[63]
	↑	↑	108	65	Yes	0.77		Plasma	TaqMan	RNU6B	[117]

miRNA			n1	n2		AUC		Sample	Method	Normalization	Ref
miR-378	↑	↓	61	61	Yes	0.861	Yes	Serum	SYBR	RNU6b	[89]
	↔		30	30	No			plasma	SYBR	cel-miR-39	[63]
	↓		14	17	No			Plasma	SYBR	Sp6	[64]
miR-421	↑		17	40	Yes	0.773		PBMC	SYBR	RNU6b	[118]
	↑		50	50	No		Yes	Serum (?)	SYBR	RNU6b	[119]
	↑		90	90	No	0.779		Serum	SYBR	RNU6b	[120]
	↑		90	90	No	0.821		PBMC	SYBR	RNU6b	[120]
miR-423-5p	↑		127	164	Yes			Serum	TaqMan	volume	[62]
	↔		30	30	No			Plasma	SYBR	cel-miR-39	[63]
miR-425	↔		58	57	Yes			Serum	TaqMan	miR-16	[49]
miR-433	↔		15	31	Yes			Serum	TaqMan	cel-miR-39	[70]
	↑	↔	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39; miR-191-5p	[68]
miR-451	↑	↓	30	56	Yes	0.96	Yes	Plasma	TaqMan	RNU6b	[54]
	↔		90	90	Yes			Plasma	SYBR	cel-miR-39	[63]
	↑	↔	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39; miR-191-5p	[68]
	↑		106	160	Yes	0.790-0.850	Yes	Plasma	TaqMan	cel-miR-39	[51]
	↔		73	206	Yes		Yes	Serum	SYBR	RNU6b	[82]
miR-484	↓	↓	14	17	No			Plasma	SYBR	Sp6	[64]
miR-486	↑	↓	30	56	Yes	0.92	Yes	Plasma	TaqMan	RNU6b	[54]
	↔		30	30	No			Plasma	SYBR	cel-miR-39	[63]
	↑		106	160	Yes	0.779-0.863	Yes	Plasma	TaqMan	cel-miR-39	[51]
	↓		14	17	No			Plasma	SYBR	Sp6	[64]
miR-501-3p	↔		73	206	Yes		Yes	Serum	SYBR	RNU6b	[82]
miR-518d	↑	↔	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39; miR-191-5p	[68]
miR-518f	↑	↔	20	20	Yes		Yes	Serum	TaqMan	cel-miR-39; miR-191-5p	[68]
miR-627	↑	↑	111	123	Yes	0.937	Yes	Plasma	SYBR	RNU6B	[81]
miR-629	↑	↑	111	123	Yes	0.912	Yes	Plasma	SYBR	RNU6B	[81]
miR-652	↑	↑	111	123	Yes	0.918	Yes	Plasma	SYBR	RNU6B	[81]
miR-720	↔		30	30	Yes			Plasma	TaqMan	RNU6b	[58]
miR-744	↑		82	82	No	0.74	Yes	Serum	TaqMan	cel-miR-39	[60]
	↔		30	30	No			Plasma	SYBR	cel-miR-39	[63]
miR-92a	↑		106	160	Yes	0.732-0.913	Yes	Plasma	TaqMan	cel-miR-39	[51]
	↓		89	92	Yes		Yes	Serum	SYBR	miR-16	[61]
miR-92b	↑	↑	109	133	Yes	0.69	Yes	Plasma	SYBR	cel-miR-39; RNU6b	[88]
miR-93	↑	↑	20	20	Yes			Plasma	TaqMan	cel-miR-39; RNU6b	[71]
	↑		65	65	Yes	0.756		Plasma	TaqMan	RNU6b	[73]
miR-940	↓	↓	105	115	Yes	0.96	Yes	Plasma	SYBR	miR-16	[55]

GC: Gastric cancer; phen/chlor: Phenol-chloroform method; SYBR: SYBR Green; NA: Non available.

miRNAs is used. This has been implemented by multiple studies and has been shown of potential diagnostic benefit independently to its scientific objectivity and validity. For instance, we analyzed miRNAs expression in ascites and showed that miR-21 was upregulated in patients with peritoneal carcinomatosis compared to control group[31]. However, also patients with peritonitis demonstrated similar increase as patients with peritoneal carcinomatosis. To overcome this limitation, we used the proportion of miR-21 (cancer-associated) and miR-223 (inflammation-associated) to differentiate the groups[31]. For miR-16, the very high values of miR-16 in erythrocytes strongly suggest that other factors such as tumor anemia or hemolysis may have an additional impact on the results. Nevertheless, this does not necessarily mean that miR-16 is not suitable, but rather that we need to know the influential factors and to know exact biogenesis of miR-16 in circulation. Further studies are needed to provide the comprehensive view on patients-related factors.

An alternative normalization way has been proposed where multiple miRNAs can be used simultaneously. For instance, miRCURY LNA Universal RT microRNA PCR System offers internal standard including miR-103a-3p, miR-191-5p, miR-423-3p and -5p and miR-451. However, we strongly doubt the usefulness of this method, as every single of those selected miRNAs have been reported as deregulated in cancer and in particular GC (Table 1). Furthermore, miR-451 is highly dependent on hemolysis and may provide some unexpected bias in analysis. Thus, in similar way as addressed for miR-16, additional studies are needed to confirm the usefulness, biological suitability and stability of the methods.

DIFFERENTIALLY EXPRESSED CIRCULATING MIRNAS IN GC PATIENTS

In the Table 1, we have summarized the miRNAs that have been analyzed for the differential expression between GC patients and controls. According to our search, we identified 106 miRNAs that were studied in different studies. Among those, 13 miRNAs such as let-
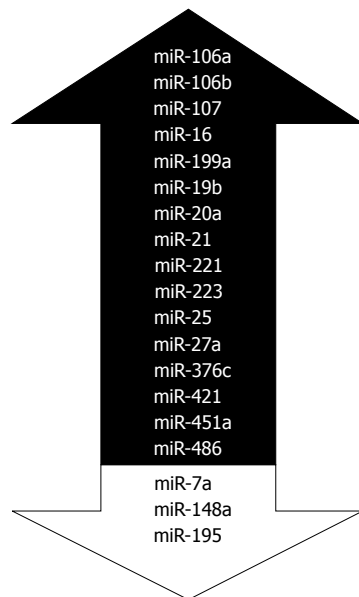
miR-106a
miR-106b
miR-107
miR-16
miR-199a
miR-19b
miR-20a
miR-21
miR-221
miR-223
miR-25
miR-27a
miR-376c
miR-421
miR-451a
miR-486

miR-7a
miR-148a
miR-195

Figure 3 Schematic presentation of most frequently deregulated microRNAs in gastric cancer. Selection was made using following criteria: at least 3 reports with at least 2 reports with either up or downregulation.

7c, let-7g, miR-143-3p, miR-122, miR-192, miR-198 etc. showed no significant changes. As shown in in Table 1, multiple miRNAs have conflicting results showing differential expression in one cohort while no changes found in another. In the Figure 3 we summarize the most frequently deregulated miRNAs. Those were selected using two criteria: (1) at least 3 publications and (2) at least two with reproducible report on increased or decreased expression. Among the most consistent miRNAs are the miR-20a, miR-223 and miR-421. Among the most studied is the miR-21 with total 13 reports. Summarizing the data to miR-21 expression, three reports showed no differences while 10 reports show an upregulation of miR-21 both in sera and in plasma of GC patients. As shown in the Table 1, multiple groups used samples from independent cohorts to confirm the results, which substantially contributes to the quality of the reports.

To estimate the diagnostic potential of miRNAs as biomarkers in GC, multiple studies provided receiver operator curves (ROC) values. For instance, Konishi *et al*[54] reported the diagnostic accuracy for miR-451 reaching 0.96 with calculated sensitivity of 96% and specificity of 100%. Liu *et al*[55] studied miR-940 and reported the ROC value of 0.96 although with slightly lower sensitivity 81.2% and specificity 98.6%. Although those results are striking, we need to keep in mind that validation of the results from independent groups or cohorts in prospective studies are still to come. We believe that the data from our summary table will be helpful for the future search, validation and discussion of the results.

PROFILING OF CIRCULATING MIRNAS IN GC PATIENTS

It is well known that the most promising way to identify

the potential biomarkers is the unbiased profiling of the samples. This approach was applied in the pivotal work by Chen *et al*[45] in 2008 in lung cancers, where the authors used multiple pooled samples for profiling and performed independent validation using qPCR. As shown in the Table 2[47,51,53-55,58,60,62,64,68,76,78,81,82,88,92,121], this approach was very intensively used for miRNA-profiling in GC patients. Among the total eighteen miRNA-profiling studies, 50% (9/18) reported the use of the pooled samples. Majority of studies used single technical pooled profiling from 5-40 samples for GC and controls. Although, this may be an appropriate way for proof-of-principle studies, it has several limitations where inappropriate changes of miRNAs in few subjects may create very strong deviation and bias and therefore independent validation is mandatory. Among the 18 profiling studies (Table 2), 11 reports studied miRNA in plasma, 6 in sera and 1 in blood samples. From the technical perspective there was substantial variation in use of extraction kits and profiling platforms used for analysis. Similar to the overall data in total cohort (Table 1), researches from China with 12 profiling studies provided the most overwhelming data. Remaining work comes from Poland, Japan, Turkey, Hong-Kong and United States. The first studies tend to use relatively small number of samples while more recent studies provide increasing number of samples and include an impressive number of samples for validation analysis as well[68,76].

FUTURE PERSPECTIVES AND CONCLUDING REMARKS

In this review, we systematically summarized and analyzed the existing data to miRNAs as non-invasive biomarkers for GC. There is no doubt that further studies with an improved study design will follow and it is a matter of time until the miRNA-based biomarkers will enter the clinical studies either in oncologic patients or patients with other diseases. However, following our critical review of the existing papers we would like to provide several cautionary notes for improvement.

Quality of reports

The era of proof-of-principle studies in GC has passed and with 75 published data it is time to improve the quality of the work. There are several ways that may be considered: (1) description of patient's cohort including Lauren's Classification, TNM staging and precise number of patients is necessary; (2) reporting of the methodological steps (extraction kits, measurements, reproducibility, validation *etc*.) need to be carefully reviewed and reported; (3) for primary research an independent cohort of samples need to be included; and (4) reporting of ROC/AUC values, sensitivity and specificity are currently used in quite biased way in proportion of GC patients to controls mostly in 1:1; however, in real-life settings the proportion will be at least 100-1000:1. Unless the prospective study is done it is clear that the real-life settings are not possible to achieve and additional effort is needed to increase the number of

Table 2 Profiling of circulating microRNAs in gastric cancer

Ref.	Year	Source	Extraction Kit	Platform	Sample Origin	GC (number of technical runs)	Controls (number of technical runs)	pooled samples	GC (number of pooled samples)	Controls (number of pooled samples)
Liu et al[62]	2011	Serum	phen/chlor	Solexa	China	1	1	Yes	20	20
Song et al[60]	2012	Serum	miRNeasy	qPCR-array	China	1	1	Yes	14	14
Liu et al[89]	2012	Serum	mirVana	Agilent	China	7	10	No		
Konishi et al[54]	2012	Plasma	mirVana	microarray	Japan	3	3	No		
Gorur et al[92]	2013	Plasma	high-pure miRNA Isolation	TaqMan Array	Turkey	1	1	Yes	20	190
Shah et al[121]	2013	Blood	miRNeasy	Geniom Biochip	Poland	1	1	Yes	8	19
Li et al[58]	2013	Plasma	mirVana	Agilent	China	20	20	No		
Zhu et al[51]	2014	Plasma	miRNeasy	TaqMan Array	China	1	1	Yes	40	40
Zhang et al[53]	2015	Plasma	mirVana	Agilent	China	16	18	No		
Zhou et al[88]	2015	Plasma	mirVana	Exiqon	China	3	1	Yes	30	10
Shin et al[81]	2015	Plasma	Trizol miRNeasy	miRCURY LNA	China	5	5	No		
Zhang et al[78]	2015	Plasma	mirVana	Agilent	China	3	3	No		
Liu et al[55]	2016	Plasma	miRNeasy	miRCURY LNA	Hong-Kong	5	5	No		
Qiu et al[47]	2016	Plasma	miRNeasy	Agilent	China	1	1	Yes	5	5
Treece et al[64]	2016	Plasma	miRCURY	GastroGenus miR Panel	United States	17	14	No		
Jiang et al[82]	2017	Serum	unknown	miSeq	China	2	1	Yes	20	10
Huang et al[76]	2017	Serum	mirVana	Exiqon	China	3	1	Yes	30	10
Sierzega et al[68]	2017	Serum	miRNA ABC Purification	TaqMan Array	Poland	20	20	No		

GC: Gastric cancer; phen/chlor: Phenol-chloroform method.

control sample.

Technical comparison between the studies

In the present review of the studies, there is a substantial technical heterogeneity among the studies: various extraction kits, qPCR methods and most importantly normalization. We have just recently published our data showing the differences between various extraction kits and miRNA expression in ascites[31]. Those results need to be taking to account and an independent validation of the primary samples will be needed. As we have recently reviewed[5], use of serum and plasma will probably have an independent effect on recovery and stability of miRNAs. Besides, contamination with various parts of circulating cells (erythrocytes, thrombocytes) may also provide an additional bias and caution in interpreting of miRNA data is necessary. As reviewed above, one of the larges differences among the studies is related to the choice of reference genes for normalization of miRNAs. Here we would like to caution the use of RNU6b for normalization of miRNAs and also probably reduce the use of miR-16 for normalization of circulating miRNAs unless additional studies provide the evidence for the objectivity of measurements.

High-risk patients

Taking to account the unique biology of GC, further focus should be made to identify the high-risk patients with the highest risk of GC development. For instance, patients with moderate to severe AG or IM are at increased risk for development of intestinal type of GC. Here, much more effort needs to be done to characterize the miRNA changes not only in GC but also in patients with preneo-plastic conditions or lesions. It would be extremely interesting to know the cascade of changes in miRNA expression in subjects with hereditary diffuse GC.

GC-heterogenic disease

In currently available studies, all GC subtypes are pooled together and most effort is made to identify "all fits one" biomarkers for GC. This is definitely the most desirable way, where all GC patients could be diagnosed with the same biomarkers; however, we need also keep in mind that GC is a heterogenic disease with unique molecular alterations[17]. Since GC shows subtype-unique miRNA alterations, it may be very likely that circulating miRNAs, at least partly, may behave differently between intestinal and diffuse types of GC. With the knowledge of TCGA molecular classification, it would also very exciting to see if different molecular subtypes have characteristic circulating miRNA expression pattern.

Beyond miRNAs: Value of isomiR's in GC

High throughput genomic data sequencing showed that conventional miRNAs may differ both in length of the molecule and its' sequence[122,123]. These variant miRNA-sequences are now being referred to as isoforms of miRNAs or isomiR's. Up to date, the role of these molecules in GC remains largely unexplored. Never-theless, increasing number of studies point out important deregulation patterns of isomiR's in cancer. For instance, in a recent study, we identified 219 deregulated isomiR'

s between gastrointestinal stromal tissue and tumor adjacent tissues[124]. To our knowledge, the only study that investigated isomiR's in GC found that certain isomiR-types preferentially occur in normal gastric tissue but other types prefer GC tissue[122]. Although these data may be available, no published data is available to isomiR's changes in blood samples of GC patients and may be focus of future studies.

Circulating tumor cells and miRNA analysis

As discussed in above, multiple studies have shown that circulating miRNAs may serve as diagnostic biomarkers for GC; however, the origin of these molecules is not fully clear. Using profiling data from sera and tumor tissues, Sierzega *et al*[68] identified differences in expression pattern in GC patients; however, miRNA expression data analysis did not support the conclusion that circulating miRNA originate primarily from the tumor tissue. Hence, it remain open if exosomal miRNAs or even certain blood-cells-related miRNAs may be more promising to study[27]. Furthermore, flow-cytometry or microfluidic-based single-cell or cell-type specific sequencing analysis may provide more specific transcription patterns with higher diagnostic and prognostic value[125]. Therefore, additional studies employing single-cell based analysis from blood samples of GC patients are needed in order to identify more sensitive and specific biomarkers.

CONCLUSION

During the past 7 years, large amount of data has been gathered to support the need for intensive research on miRNA-based biomarkers in GC. In this review, we systematically summarized the data to miRNAs as non-invasive diagnostic biomarkers in GC. Meticulous analysis of the published work revealed relatively high level of heterogeneity not only in methodological and technical aspects, but also in reporting quality of the studies. At present, no miRNA-based biomarkers are ready to be implemented for GC-screening other than in research studies; however, the provided data clearly highlight the potential of miRNAs as diagnostic biomarkers and also highlight the need for the further improvement. We hope that this first of its kind comprehensive review with critical points may lead to a "next wave" of "second generation" comprehensive studies that take into account not only technical aspects of miRNA research but also unique aspects of GC biology.

REFERENCES

1   **Chen W**, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. *CA Cancer J Clin* 2016; **66**: 115-132 [PMID: 26808342 DOI: 10.3322/caac.21338]

2   **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* 2018; **68**: 7-30 [PMID: 29313949 DOI: 10.3322/caac.21442]

3   **Lu J**, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. *Nature* 2005; **435**: 834-838 [PMID: 15944708 DOI: 10.1038/nature03702]

4   **Link A**, Kupcinskas J, Wex T, Malfertheiner P. Macro-role of microRNA in gastric cancer. *Dig Dis* 2012; **30**: 255-267 [PMID: 22722550 DOI: 10.1159/000336919]

5   **Link A**, Goel A. MicroRNA in gastrointestinal cancer: a step closer to reality. *Adv Clin Chem* 2013; **62**: 221-268 [PMID: 24772669 DOI: 10.1016/B978-0-12-800096-0.00006-8]

6   **Sugano K**, Tack J, Kuipers EJ, Graham DY, El-Omar EM, Miura S, Haruma K, Asaka M, Uemura N, Malfertheiner P; faculty members of Kyoto Global Consensus Conference. Kyoto global consensus report on Helicobacter pylori gastritis. *Gut* 2015; **64**: 1353-1367 [PMID: 26187502 DOI: 10.1136/gutjnl-2015-309252]

7   **Malfertheiner P**, Link A, Selgrad M. Helicobacter pylori: perspectives and time trends. *Nat Rev Gastroenterol Hepatol* 2014; **11**: 628-638 [PMID: 25001975 DOI: 10.1038/nrgastro.2014.99]

8   **Bornschein J**, Leja M, Kupcinskas J, Link A, Weaver J, Rugge M, Malfertheiner P. Molecular diagnostics in gastric cancer. *Front Biosci (Landmark Ed)* 2014; **19**: 312-338 [PMID: 24389187 DOI: 10.2741/4210]

9   **Chiba T**, Marusawa H, Ushijima T. Inflammation-associated cancer development in digestive organs: mechanisms and roles for genetic and epigenetic modulation. *Gastroenterology* 2012; **143**: 550-563 [PMID: 22796521 DOI: 10.1053/j.gastro.2012.07.009]

10   **Song H**, Ekheden IG, Zheng Z, Ericsson J, Nyrén O, Ye W. Incidence of gastric cancer among patients with gastric precancerous lesions: observational cohort study in a low risk Western population. *BMJ* 2015; **351**: h3867 [PMID: 26215280 DOI: 10.1136/bmj.h3867]

11   **Beardwell I**, Holden L, Claydon T. Human resource management: a contemporary approach [Internet]. 2004

12   **Atherton JC**. The pathogenesis of Helicobacter pylori-induced gastro-duodenal diseases. *Annu Rev Pathol* 2006; **1**: 63-96 [PMID: 18039108 DOI: 10.1146/annurev.pathol.1.110304.100125]

13   **Basso D**, Zambon CF, Letley DP, Stranges A, Marchet A, Rhead JL, Schiavon S, Guariso G, Ceroti M, Nitti D, Rugge M, Plebani M, Atherton JC. Clinical relevance of Helicobacter pylori cagA and vacA gene polymorphisms. *Gastroenterology* 2008; **135**: 91-99 [PMID: 18474244 DOI: 10.1053/j.gastro.2008.03.041]

14   **Link A**, Langner C, Schirrmeister W, Habendorf W, Weigt J, Venerito M, Tammer I, Schlüter D, Schlaermann P, Meyer TF, Wex T, Malfertheiner P. *Helicobacter pylori* vacA genotype is a predominant determinant of immune response to *Helicobacter pylori* CagA. *World J Gastroenterol* 2017; **23**: 4712-4723 [PMID: 28765692 DOI: 10.3748/wjg.v23.i26.4712]

15   **Malfertheiner P**, Megraud F, O'Morain CA, Gisbert JP, Kuipers EJ, Axon AT, Bazzoli F, Gasbarrini A, Atherton J, Graham DY, Hunt R, Moayyedi P, Rokkas T, Rugge M, Selgrad M, Suerbaum S, Sugano K, El-Omar EM; European Helicobacter and Microbiota Study Group and Consensus panel. Management of Helicobacter pylori infection-the Maastricht V/Florence Consensus Report. *Gut* 2017; **66**: 6-30 [PMID: 27707777 DOI: 10.1136/gutjnl-2016-312288]

16   **Lauren P**. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. *Acta Pathol Microbiol Scand* 1965; **64**: 31-49 [PMID: 14320675 DOI: 10.1111/apm.1965.64.1.31]

17   **Cancer Genome Atlas Research Network**. Comprehensive molecular characterization of gastric adenocarcinoma. *Nature* 2014; **513**: 202-209 [PMID: 25079317 DOI: 10.1038/nature13480]

18   **Shimada H**, Noie T, Ohashi M, Oba K, Takahashi Y. Clinical significance of serum tumor markers for gastric cancer: a systematic review of literature by the Task Force of the Japanese Gastric Cancer Association. *Gastric Cancer* 2014; **17**: 26-33 [PMID: 23572188 DOI: 10.1007/s10120-013-0259-5]

19   **Goni E**, Venerito M, Schulz C, Weigt J, Langner C, Link A, Malfertheiner P. Influence of laboratory-related and endoscopy-related factors on the assessment of serum pepsinogens and gastrin-17. *Eur J Gastroenterol Hepatol* 2017; **29**: 1340-1345 [PMID: 28984679 DOI: 10.1097/MEG.0000000000000952]

20   **Zagari RM**, Rabitti S, Greenwood DC, Eusebi LH, Vestito A, Bazzoli F. Systematic review with meta-analysis: diagnostic performance of the combination of pepsinogen, gastrin-17 and

anti-Helicobacter pylori antibodies serum assays for the diagnosis of atrophic gastritis. *Aliment Pharmacol Ther* 2017; **46**: 657-667 [PMID: 28782119 DOI: 10.1111/apt.14248]

21   **Huang YK**, Yu JC, Kang WM, Ma ZQ, Ye X, Tian SB, Yan C. Significance of Serum Pepsinogens as a Biomarker for Gastric Cancer and Atrophic Gastritis Screening: A Systematic Review and Meta-Analysis. *PLoS One* 2015; **10**: e0142080 [PMID: 26556485 DOI: 10.1371/journal.pone.0142080]

22   **Bornschein J**, Selgrad M, Wex T, Kuester D, Malfertheiner P. Serological assessment of gastric mucosal atrophy in gastric cancer. *BMC Gastroenterol* 2012; **12**: 10 [PMID: 22289789 DOI: 10.1186/1471-230X-12-10]

23   **Lujambio A**, Lowe SW. The microcosmos of cancer. *Nature* 2012; **482**: 347-355 [PMID: 22337054 DOI: 10.1038/nature10888]

24   **McLean MH**, El-Omar EM. Genetics of gastric cancer. *Nat Rev Gastroenterol Hepatol* 2014; **11**: 664-674 [PMID: 25134511 DOI: 10.1038/nrgastro.2014.143]

25   **Link A**, Schirrmeister W, Langner C, Varbanova M, Bornschein J, Wex T, Malfertheiner P. Differential expression of microRNAs in preneoplastic gastric mucosa. *Sci Rep* 2015; **5**: 8270 [PMID: 25652892 DOI: 10.1038/srep08270]

26   **Juzènas S**, Saltenienè V, Kupcinskas J, Link A, Kiudelis G, Jonaitis L, Jarmalaite S, Kupcinskas L, Malfertheiner P, Skieceviciene J. Analysis of Deregulated microRNAs and Their Target Genes in Gastric Cancer. *PLoS One* 2015; **10**: e0132327 [PMID: 26172537 DOI: 10.1371/journal.pone.0132327]

27   **Juzenas S**, Venkatesh G, Hübenthal M, Hoeppner MP, Du ZG, Paulsen M, Rosenstiel P, Senger P, Hofmann-Apitius M, Keller A, Kupcinskas L, Franke A, Hemmrich-Stanisak G. A comprehensive, cell specific microRNA catalogue of human peripheral blood. *Nucleic Acids Res* 2017; **45**: 9290-9301 [PMID: 28934507 DOI: 10.1093/nar/gkx706]

28   **Link A**, Balaguer F, Shen Y, Nagasaka T, Lozano JJ, Boland CR, Goel A. Fecal MicroRNAs as novel biomarkers for colon cancer screening. *Cancer Epidemiol Biomarkers Prev* 2010; **19**: 1766-1774 [PMID: 20551304 DOI: 10.1158/1055-9965.EPI-10-0027]

29   **Link A**, Becker V, Goel A, Wex T, Malfertheiner P. Feasibility of fecal microRNAs as novel biomarkers for pancreatic cancer. *PLoS One* 2012; **7**: e42933 [PMID: 22905187 DOI: 10.1371/journal.pone.0042933]

30   **Yu L**, Todd NW, Xing L, Xie Y, Zhang H, Liu Z, Fang H, Zhang J, Katz RL, Jiang F. Early detection of lung adenocarcinoma in sputum by a panel of microRNA markers. *Int J Cancer* 2010; **127**: 2870-2878 [PMID: 21351266 DOI: 10.1002/ijc.25289]

31   **Schindler P**, Kupcinskas J, Juzenas S, Skieceviciene J, Salteniene V, Schulz C, Weigt J, Malfertheiner P, Link A. Expression of microRNAs in the ascites of patients with peritoneal carcinomatosis and peritonitis. *Cancer Cytopathol* 2018; **126**: 353-363 [PMID: 29360196 DOI: 10.1002/cncy.21966]

32   **Weber JA**, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, Galas DJ, Wang K. The microRNA spectrum in 12 body fluids. *Clin Chem* 2010; **56**: 1733-1741 [PMID: 20847327 DOI: 10.1373/clinchem.2010.147405]

33   **Ueda T**, Volinia S, Okumura H, Shimizu M, Taccioli C, Rossi S, Alder H, Liu CG, Oue N, Yasui W, Yoshida K, Sasaki H, Nomura S, Seto Y, Kaminishi M, Calin GA, Croce CM. Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis. *Lancet Oncol* 2010; **11**: 136-146 [PMID: 20022810 DOI: 10.1016/S1470-2045(09)70343-2]

34   **Steponaitiene R**, Kupcinskas J, Langner C, Balaguer F, Venclauskas L, Pauzas H, Tamelis A, Skieceviciene J, Kupcinskas L, Malfertheiner P, Link A. Epigenetic silencing of miR-137 is a frequent event in gastric carcinogenesis. *Mol Carcinog* 2016; **55**: 376-386 [PMID: 25663388 DOI: 10.1002/mc.22287]

35   **Han TS**, Hur K, Xu G, Choi B, Okugawa Y, Toiyama Y, Oshima H, Oshima M, Lee HJ, Kim VN, Chang AN, Goel A, Yang HK. MicroRNA-29c mediates initiation of gastric carcinogenesis by directly targeting ITGB1. *Gut* 2015; **64**: 203-214 [PMID: 24870620 DOI: 10.1136/gutjnl-2013-306640]

36   **Sousa JF**, Nam KT, Petersen CP, Lee HJ, Yang HK, Kim WH, Goldenring JR. miR-30-HNF4γ and miR-194-NR2F2 regulatory networks contribute to the upregulation of metaplasia markers in the stomach. *Gut* 2016; **65**: 914-924 [PMID: 25800782 DOI: 10.1136/gutjnl-2014-308759]

37   **Mishra PJ**, Bertino JR. MicroRNA polymorphisms: the future of pharmacogenomics, molecular epidemiology and individualized medicine. *Pharmacogenomics* 2009; **10**: 399-416 [PMID: 19290790 DOI: 10.2217/14622416.10.3.399]

38   **Liu C**, Rennie WA, Carmack CS, Kanoria S, Cheng J, Lu J, Ding Y. Effects of genetic variations on microRNA: target interactions. *Nucleic Acids Res* 2014; **42**: 9543-9552 [PMID: 25081214 DOI: 10.1093/nar/gku675]

39   **Kupcinskas J**, Bruzaite I, Juzenas S, Gyvyte U, Jonaitis L, Kiudelis G, Skieceviciene J, Leja M, Pauzas H, Tamelis A, Pavalkis D, Kupcinskas L. Lack of association between miR-27a, miR-146a, miR-196a-2, miR-492 and miR-608 gene polymorphisms and colorectal cancer. *Sci Rep* 2014; **4**: 5993 [PMID: 25103961 DOI: 10.1038/srep05993]

40   **Kupcinskas J**, Wex T, Link A, Leja M, Bruzaite I, Steponaitiene R, Juzenas S, Gyvyte U, Ivanauskas A, Ancans G, Petrenkiene V, Skieceviciene J, Kupcinskas L, Malfertheiner P. Gene polymorphisms of micrornas in Helicobacter pylori-induced high risk atrophic gastritis and gastric cancer. *PLoS One* 2014; **9**: e87467 [PMID: 24475294 DOI: 10.1371/journal.pone.0087467]

41   **Petkevicius V**, Salteniene V, Juzenas S, Wex T, Link A, Leja M, Steponaitiene R, Skieceviciene J, Kupcinskas L, Jonaitis L, Kiudelis G, Malfertheiner P, Kupcinskas J. Polymorphisms of microRNA target genes *IL12B*, *INSR*, *CCND1* and *IL10* in gastric cancer. *World J Gastroenterol* 2017; **23**: 3480-3487 [PMID: 28596683 DOI: 10.3748/wjg.v23.i19.3480]

42   **Fu B**, Song P, Lu M, Wang B, Zhao Q. The association between miR-146a gene rs2910164 polymorphism and gastric cancer risk: a meta-analysis. *Biomed Pharmacother* 2014; **68**: 923-928 [PMID: 25455160 DOI: 10.1016/j.biopha.2014.10.002]

43   **Lawrie CH**, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, Banham AH, Pezzella F, Boultwood J, Wainscoat JS, Hatton CS, Harris AL. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. *Br J Haematol* 2008; **141**: 672-675 [PMID: 18318758 DOI: 10.1111/j.1365-2141.2008.07077.x]

44   **Mitchell PS**, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* 2008; **105**: 10513-10518 [PMID: 18663219 DOI: 10.1073/pnas.0804549105]

45   **Chen X**, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, Li Q, Li X, Wang W, Zhang Y, Wang J, Jiang X, Xiang Y, Xu C, Zheng P, Zhang J, Li R, Zhang H, Shang X, Gong T, Ning G, Wang J, Zen K, Zhang J, Zhang CY. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res* 2008; **18**: 997-1006 [PMID: 18766170 DOI: 10.1038/cr.2008.282]

46   **Tokuhisa M**, Ichikawa Y, Kosaka N, Ochiya T, Yashiro M, Hirakawa K, Kosaka T, Makino H, Akiyama H, Kunisaki C, Endo I. Exosomal miRNAs from Peritoneum Lavage Fluid as Potential Prognostic Biomarkers of Peritoneal Metastasis in Gastric Cancer. *PLoS One* 2015; **10**: e0130472 [PMID: 26208314 DOI: 10.1371/journal.pone.0130472]

47   **Qiu X**, Zhang J, Shi W, Liu S, Kang M, Chu H, Wu D, Tong N, Gong W, Tao G, Zhao Q, Qiang F, Zhu H, Wu Q, Wang M, Zhang Z. Circulating MicroRNA-26a in Plasma and Its Potential Diagnostic Value in Gastric Cancer. *PLoS One* 2016; **11**: e0151345 [PMID: 27010210 DOI: 10.1371/journal.pone.0151345]

48   **Song J**, Bai Z, Han W, Zhang J, Meng H, Bi J, Ma X, Han S, Zhang Z. Identification of suitable reference genes for qPCR analysis of serum microRNA in gastric cancer patients. *Dig Dis Sci* 2012; **57**: 897-904 [PMID: 22198701 DOI: 10.1007/s10620-011-1981-7]

49   **Peng WZ**, Ma R, Wang F, Yu J, Liu ZB. Role of miR-191/425

cluster in tumorigenesis and diagnosis of gastric cancer. *Int J Mol Sci* 2014; **15**: 4031-4048 [PMID: 24603541 DOI: 10.3390/ijms15034031]

50   **Shiotani A**, Murao T, Kimura Y, Matsumoto H, Kamada T, Kusunoki H, Inoue K, Uedo N, Iishi H, Haruma K. Identification of serum miRNAs as novel non-invasive biomarkers for detection of high risk for early gastric cancer. *Br J Cancer* 2013; **109**: 2323-2330 [PMID: 24104965 DOI: 10.1038/bjc.2013.596]

51   **Zhu C**, Ren C, Han J, Ding Y, Du J, Dai N, Dai J, Ma H, Hu Z, Shen H, Xu Y, Jin G. A five-microRNA panel in plasma was identified as potential biomarker for early detection of gastric cancer. *Br J Cancer* 2014; **110**: 2291-2299 [PMID: 24595006 DOI: 10.1038/bjc.2014.119]

52   **Wang H**, Wang L, Wu Z, Sun R, Jin H, Ma J, Liu L, Ling R, Yi J, Wang L, Bian J, Chen J, Li N, Yuan S, Yun J. Three dysregulated microRNAs in serum as novel biomarkers for gastric cancer screening. *Med Oncol* 2014; **31**: 298 [PMID: 25367852 DOI: 10.1007/s12032-014-0298-8]

53   **Zhang J**, Song Y, Zhang C, Zhi X, Fu H, Ma Y, Chen Y, Pan F, Wang K, Ni J, Jin W, He X, Su H, Cui D. Circulating MiR-16-5p and MiR-19b-3p as Two Novel Potential Biomarkers to Indicate Progression of Gastric Cancer. *Theranostics* 2015; **5**: 733-745 [PMID: 25897338 DOI: 10.7150/thno.10305]

54   **Konishi H**, Ichikawa D, Komatsu S, Shiozaki A, Tsujiura M, Takeshita H, Morimura R, Nagata H, Arita T, Kawaguchi T, Hirashima S, Fujiwara H, Okamoto K, Otsuji E. Detection of gastric cancer-associated microRNAs on microRNA microarray comparing pre- and post-operative plasma. *Br J Cancer* 2012; **106**: 740-747 [PMID: 22262318 DOI: 10.1038/bjc.2011.588]

55   **Liu X**, Kwong A, Sihoe A, Chu KM. Plasma miR-940 may serve as a novel biomarker for gastric cancer. *Tumour Biol* 2016; **37**: 3589-3597 [PMID: 26456959 DOI: 10.1007/s13277-015-4019-5]

56   **Tsujiura M**, Ichikawa D, Komatsu S, Shiozaki A, Takeshita H, Kosuga T, Konishi H, Morimura R, Deguchi K, Fujiwara H, Okamoto K, Otsuji E. Circulating microRNAs in plasma of patients with gastric cancers. *Br J Cancer* 2010; **102**: 1174-1179 [PMID: 20234369 DOI: 10.1038/sj.bjc.6605608]

57   **Wang WN**, Chen Y, Zhang YD, Hu TH. The regulatory mechanism of CCR7 gene expression and its involvement in the metastasis and progression of gastric cancer. *Tumour Biol* 2013; **34**: 1865-1871 [PMID: 23519840 DOI: 10.1007/s13277-013-0728-9]

58   **Li C**, Li JF, Cai Q, Qiu QQ, Yan M, Liu BY, Zhu ZG. miRNA-199a-3p in plasma as a potential diagnostic biomarker for gastric cancer. *Ann Surg Oncol* 2013; **20** Suppl 3: S397-S405 [PMID: 22956063 DOI: 10.1245/s10434-012-2600-3]

59   **Liu WJ**, Xu Q, Sun LP, Dong QG, He CY, Yuan Y. Expression of serum let-7c, let-7i, and let-7f microRNA with its target gene, pepsinogen C, in gastric cancer and precancerous disease. *Tumour Biol* 2015; **36**: 3337-3343 [PMID: 25549793 DOI: 10.1007/s13277-014-2967-9]

60   **Song MY**, Pan KF, Su HJ, Zhang L, Ma JL, Li JY, Yuasa Y, Kang D, Kim YS, You WC. Identification of serum microRNAs as novel non-invasive biomarkers for early detection of gastric cancer. *PLoS One* 2012; **7**: e33608 [PMID: 22432036 DOI: 10.1371/journal.pone.0033608]

61   **Huang S**, Wang J, Li J, Luo Q, Zhao M, Zheng L, Dong X, Chen C, Che Y, Liu P, Qi J, Huang C. Serum microRNA expression profile as a diagnostic panel for gastric cancer. *Jpn J Clin Oncol* 2016; **46**: 811-818 [PMID: 27380811 DOI: 10.1093/jjco/hyw085]

62   **Liu R**, Zhang C, Hu Z, Li G, Wang C, Yang C, Huang D, Chen X, Zhang H, Zhuang R, Deng T, Liu H, Yin J, Wang S, Zen K, Ba Y, Zhang CY. A five-microRNA signature identified from genome-wide serum microRNA expression profiling serves as a fingerprint for gastric cancer diagnosis. *Eur J Cancer* 2011; **47**: 784-791 [PMID: 21112772 DOI: 10.1016/j.ejca.2010.10.025]

63   **Cai H**, Yuan Y, Hao YF, Guo TK, Wei X, Zhang YM. Plasma microRNAs serve as novel potential biomarkers for early detection of gastric cancer. *Med Oncol* 2013; **30**: 452 [PMID: 23307259 DOI: 10.1007/s12032-012-0452-0]

64   **Treece AL**, Duncan DL, Tang W, Elmore S, Morgan DR, Dominguez RL, Speck O, Meyers MO, Gulley ML. Gastric adenocarcinoma microRNA profiles in fixed tissue and in plasma reveal cancer-associated and Epstein-Barr virus-related expression patterns. *Lab Invest* 2016; **96**: 661-671 [PMID: 26950485 DOI: 10.1038/labinvest.2016.33]

65   **Oze I**, Shimada S, Nagasaki H, Akiyama Y, Watanabe M, Yatabe Y, Matsuo K, Yuasa Y. Plasma microRNA-103, microRNA-107, and microRNA-194 levels are not biomarkers for human diffuse gastric cancer. *J Cancer Res Clin Oncol* 2017; **143**: 551-554 [PMID: 27909811 DOI: 10.1007/s00432-016-2316-z]

66   **Zhou H**, Guo JM, Lou YR, Zhang XJ, Zhong FD, Jiang Z, Cheng J, Xiao BX. Detection of circulating tumor cells in peripheral blood from patients with gastric cancer using microRNA as a marker. *J Mol Med (Berl)* 2010; **88**: 709-717 [PMID: 20349219 DOI: 10.1007/s00109-010-0617-2]

67   **Yuan R**, Wang G, Xu Z, Zhao H, Chen H, Han Y, Wang B, Zhou J, Hu H, Guo Z, Shen H, Xue X. Up-regulated Circulating miR-106a by DNA Methylation Promised a Potential Diagnostic and Prognostic Marker for Gastric Cancer. *Anticancer Agents Med Chem* 2016; **16**: 1093-1100 [PMID: 26179261 DOI: 10.2174/1871520615666150716110657]

68   **Sierzega M**, Kaczor M, Kolodziejczyk P, Kulig J, Sanak M, Richter P. Evaluation of serum microRNA biomarkers for gastric cancer based on blood and tissue pools profiling: the importance of miR-21 and miR-331. *Br J Cancer* 2017; **117**: 266-273 [PMID: 28641313 DOI: 10.1038/bjc.2017.190]

69   **Wang N**, Wang L, Yang Y, Gong L, Xiao B, Liu X. A serum exosomal microRNA panel as a potential biomarker test for gastric cancer. *Biochem Biophys Res Commun* 2017; **493**: 1322-1328 [PMID: 28986250 DOI: 10.1016/j.bbrc.2017.10.003]

70   **Kim SY**, Jeon TY, Choi CI, Kim DH, Kim DH, Kim GH, Ryu DY, Lee BE, Kim HH. Validation of circulating miRNA biomarkers for predicting lymph node metastasis in gastric cancer. *J Mol Diagn* 2013; **15**: 661-669 [PMID: 23806809 DOI: 10.1016/j.jmoldx.2013.04.004]

71   **Zhang R**, Wang W, Li F, Zhang H, Liu J. MicroRNA-106b~25 expressions in tumor tissues and plasma of patients with gastric cancers. *Med Oncol* 2014; **31**: 243 [PMID: 25218271 DOI: 10.1007/s12032-014-0243-x]

72   **Zeng Q**, Jin C, Chen W, Xia F, Wang Q, Fan F, Du J, Guo Y, Lin C, Yang K, Li J, Peng X, Li X, Cao K. Downregulation of serum miR-17 and miR-106b levels in gastric cancer and benign gastric diseases. *Chin J Cancer Res* 2014; **26**: 711-716 [PMID: 25561770 DOI: 10.3978/j.issn.1000-9604.2014.12.03]

73   **Li F**, Guo Y, Liu J, Zhang R. The significance of elevated plasma expression of microRNA 106b~25 clusters in gastric cancer. *PLoS One* 2017; **12**: e0178427 [PMID: 28562634 DOI: 10.1371/journal.pone.0178427]

74   **Ayremlou N**, Mozdarani H, Mowla SJ, Delavari A. Increased levels of serum and tissue miR-107 in human gastric cancer: Correlation with tumor hypoxia. *Cancer Biomark* 2015; **15**: 851-860 [PMID: 26406411 DOI: 10.3233/CBM-150529]

75   **Zhang M**, Wang X, Li W, Cui Y. miR-107 and miR-25 simultaneously target LATS2 and regulate proliferation and invasion of gastric adenocarcinoma (GAC) cells. *Biochem Biophys Res Commun* 2015; **460**: 806-812 [PMID: 25824045 DOI: 10.1016/j.bbrc.2015.03.110]

76   **Huang Z**, Zhu D, Wu L, He M, Zhou X, Zhang L, Zhang H, Wang W, Zhu J, Cheng W, Chen Y, Fan Y, Qi L, Yin Y, Zhu W, Shu Y, Liu P. Six Serum-Based miRNAs as Potential Diagnostic Biomarkers for Gastric Cancer. *Cancer Epidemiol Biomarkers Prev* 2017; **26**: 188-196 [PMID: 27756776 DOI: 10.1158/1055-9965.EPI-16-0607]

77   **Chen Q**, Ge X, Zhang Y, Xia H, Yuan D, Tang Q, Chen L, Pang X, Leng W, Bi F. Plasma miR-122 and miR-192 as potential novel biomarkers for the early detection of distant metastasis of gastric cancer. *Oncol Rep* 2014; **31**: 1863-1870 [PMID: 24481716 DOI: 10.3892/or.2014.3004]

78   **Zhang ZZ**, Wang CJ, Niu L, Xu J, Wang M, Cao H, Hu B. Analysis of plasma MicroRNAs to identifying early diagnostic molecule for gastric cancer. *Int J Clin Exp Med* 2015; **8**: 3700-3706 [PMID: 26064265]

79   **Jiang H**, Yu WW, Wang LL, Peng Y. miR-130a acts as a potential diagnostic biomarker and promotes gastric cancer migration, invasion and proliferation by targeting RUNX3. *Oncol Rep* 2015; **34**: 1153-1161 [PMID: 26134263 DOI: 10.3892/or.2015.4099]

80   **Chen S**, Zhu J, Yu F, Tian Y, Ma S, Liu X. Combination of miRNA and RNA functions as potential biomarkers for gastric cancer. *Tumour Biol* 2015; **36**: 9909-9918 [PMID: 26168960 DOI: 10.1007/s13277-015-3756-9]

81   **Shin VY**, Ng EK, Chan VW, Kwong A, Chu KM. A three-miRNA signature as promising non-invasive diagnostic marker for gastric cancer. *Mol Cancer* 2015; **14**: 202 [PMID: 26607322 DOI: 10.1186/s12943-015-0473-3]

82   **Jiang X**, Wang W, Yang Y, Du L, Yang X, Wang L, Zheng G, Duan W, Wang R, Zhang X, Wang L, Chen X, Wang C. Identification of circulating microRNA signatures as potential noninvasive biomarkers for prediction and prognosis of lymph node metastasis in gastric cancer. *Oncotarget* 2017;724:65132-65142 [DOI: 10.18632/oncotarget.17789]

83   **Liu S**, Suo J, Wang C, Sun X, Wang D, He L, Zhang Y, Li W. Prognostic significance of low miR-144 expression in gastric cancer. *Cancer Biomark* 2017; **20**: 547-552 [PMID: 28800316 DOI: 10.3233/CBM-170351]

84   **Tang G**, Shen X, Lv K, Wu Y, Bi J, Shen Q. Different normalization strategies might cause inconsistent variation in circulating microRNAs in patients with hepatocellular carcinoma. *Med Sci Monit* 2015; **21**: 617-624 [PMID: 25719241 DOI: 10.12659/MSM.891028]

85   **Park JL**, Kim M, Song KS, Kim SY, Kim YS. Cell-Free miR-27a, a Potential Diagnostic and Prognostic Biomarker for Gastric Cancer. *Genomics Inform* 2015; **13**: 70-75 [PMID: 26523130 DOI: 10.5808/GI.2015.13.3.70]

86   **Zhao C**, Li Y, Chen G, Wang F, Shen Z, Zhou R. Overexpression of miR-15b-5p promotes gastric cancer metastasis by regulating PAQR3. *Oncol Rep* 2017; **38**: 352-358 [PMID: 28560431 DOI: 10.3892/or.2017.5673]

87   **Cui MH**, Hou XL, Lei XY, Mu FH, Yang GB, Yue L, Fu Y, Yi GX. Upregulation of microRNA 181c expression in gastric cancer tissues and plasma. *Asian Pac J Cancer Prev* 2013; **14**: 3063-3066 [PMID: 23803080 DOI: 10.7314/APJCP.2013.14.5.3063]

88   **Zhou X**, Zhu W, Li H, Wen W, Cheng W, Wang F, Wu Y, Qi L, Fan Y, Chen Y, Ding Y, Xu J, Qian J, Huang Z, Wang T, Zhu D, Shu Y, Liu P. Diagnostic value of a plasma microRNA signature in gastric cancer: a microRNA expression analysis. *Sci Rep* 2015; **5**: 11251 [PMID: 26059512 DOI: 10.1038/srep11251]

89   **Liu H**, Zhu L, Liu B, Yang L, Meng X, Zhang W, Ma Y, Xiao H. Genome-wide microRNA profiles identify miR-378 as a serum biomarker for early detection of gastric cancer. *Cancer Lett* 2012; **316**: 196-203 [PMID: 22169097 DOI: 10.1016/j.canlet.2011.10.034]

90   **Tsujiura M**, Komatsu S, Ichikawa D, Shiozaki A, Konishi H, Takeshita H, Moriumura R, Nagata H, Kawaguchi T, Hirajima S, Arita T, Fujiwara H, Okamoto K, Otsuji E. Circulating miR-18a in plasma contributes to cancer detection and monitoring in patients with gastric cancer. *Gastric Cancer* 2015; **18**: 271-279 [PMID: 24626859 DOI: 10.1007/s10120-014-0363-1]

91   **Shen YH**, Xie ZB, Yue AM, Wei QD, Zhao HF, Yin HD, Mai W, Zhong XG, Huang SR. Expression level of microRNA-195 in the serum of patients with gastric cancer and its relationship with the clinicopathological staging of the cancer. *Eur Rev Med Pharmacol Sci* 2016; **20**: 1283-1287 [PMID: 27097947]

92   **Gorur A**, Balci Fidanci S, Dogruer Unal N, Ayaz L, Akbayir S, Yildirim Yaroglu H, Dirlik M, Serin MS, Tamer L. Determination of plasma microRNA for early detection of gastric cancer. *Mol Biol Rep* 2013; **40**: 2091-2096 [PMID: 23212612 DOI: 10.1007/s11033-012-2267-7]

93   **Tsai MM**, Wang CS, Tsai CY, Huang CG, Lee KF, Huang HW, Lin YH, Chi HC, Kuo LM, Lu PH, Lin KH. Circulating microRNA-196a/b are novel biomarkers associated with metastatic gastric cancer. *Eur J Cancer* 2016; **64**: 137-148 [PMID: 27420607 DOI: 10.1016/j.ejca.2016.05.007]

94   **Li C**, Li JF, Cai Q, Qiu QQ, Yan M, Liu BY, Zhu ZG. MiRNA-199a-3p: A potential circulating diagnostic biomarker for early gastric cancer. *J Surg Oncol* 2013; **108**: 89-92 [PMID: 23733518 DOI: 10.1002/jso.23358]

95   **Valladares-Ayerbes M**, Reboredo M, Medina-Villaamil V, Iglesias-Díaz P, Lorenzo-Patiño MJ, Haz M, Santamarina I, Blanco M, Fernández-Tajes J, Quindós M, Carral A, Figueroa A, Antón-Aparicio LM, Calvo L. Circulating miR-200c as a diagnostic and prognostic biomarker for gastric cancer. *J Transl Med* 2012; **10**: 186 [PMID: 22954417 DOI: 10.1186/1479-5876-10-186]

96   **Zhang HP**, Sun FB, Li SJ. Serum miR-200c expression level as a prognostic biomarker for gastric cancer. *Genet Mol Res* 2015; **14**: 15913-15920 [PMID: 26662382 DOI: 10.4238/2015.December.7.2]

97   **Imaoka H**, Toiyama Y, Okigami M, Yasuda H, Saigusa S, Ohi M, Tanaka K, Inoue Y, Mohri Y, Kusunoki M. Circulating microRNA-203 predicts metastases, early recurrence, and poor prognosis in human gastric cancer. *Gastric Cancer* 2016; **19**: 744-753 [PMID: 26233325 DOI: 10.1007/s10120-015-0521-0]

98   **Chen X**, Liu XS, Liu HY, Lu YY, Li Y. Reduced expression of serum miR-204 predicts poor prognosis of gastric cancer. *Genet Mol Res* 2016; **15**: [PMID: 27173244 DOI: 10.4238/gmr.15027702]

99   **Hou CG**, Luo XY, Li G. Diagnostic and Prognostic Value of Serum MicroRNA-206 in Patients with Gastric Cancer. *Cell Physiol Biochem* 2016; **39**: 1512-1520 [PMID: 27614739 DOI: 10.1159/000447854]

100   **Du Y**, Zhu M, Zhou X, Huang Z, Zhu J, Xu J, Cheng G, Shu Y, Liu P, Zhu W, Wang T. miR-20a enhances cisplatin resistance of human gastric cancer cell line by targeting NFKBIB. *Tumour Biol* 2016; **37**: 1261-1269 [PMID: 26286834 DOI: 10.1007/s13277-015-3921-1]

101   **Zhu M**, Zhou X, Du Y, Huang Z, Zhu J, Xu J, Cheng G, Shu Y, Liu P, Zhu W, Wang T. miR-20a induces cisplatin resistance of a human gastric cancer cell line via targeting CYLD. *Mol Med Rep* 2016; **14**: 1742-1750 [PMID: 27357419 DOI: 10.3892/mmr.2016.5413]

102   **Yang R**, Fu Y, Zeng Y, Xiang M, Yin Y, Li L, Xu H, Zhong J, Zeng X. Serum miR-20a is a promising biomarker for gastric cancer. *Biomed Rep* 2017; **6**: 429-434 [PMID: 28413641 DOI: 10.3892/br.2017.862]

103   **Zheng Y**, Cui L, Sun W, Zhou H, Yuan X, Huo M, Chen J, Lou Y, Guo J. MicroRNA-21 is a new marker of circulating tumor cells in gastric cancer patients. *Cancer Biomark* 2011; **10**: 71-77 [PMID: 22430134 DOI: 10.3233/CBM-2011-0231]

104   **Li BS**, Zhao YL, Guo G, Li W, Zhu ED, Luo X, Mao XH, Zou QM, Yu PW, Zuo QF, Li N, Tang B, Liu KY, Xiao B. Plasma microRNAs, miR-223, miR-21 and miR-218, as novel potential biomarkers for gastric cancer detection. *PLoS One* 2012; **7**: e41629 [PMID: 22860003 DOI: 10.1371/journal.pone.0041629]

105   **Wang B**, Zhang Q. The expression and clinical significance of circulating microRNA-21 in serum of five solid tumors. *J Cancer Res Clin Oncol* 2012; **138**: 1659-1666 [PMID: 22638884 DOI: 10.1007/s00432-012-1244-9]

106   **Wu J**, Li G, Wang Z, Yao Y, Chen R, Pu X, Wang J. Circulating MicroRNA-21 Is a Potential Diagnostic Biomarker in Gastric Cancer. *Dis Markers* 2015; **2015**: 435656 [PMID: 26063956 DOI: 10.1155/2015/435656]

107   **Xin SY**, Feng XS, Zhou LQ, Sun JJ, Gao XL, Yao GL. Reduced expression of circulating microRNA-218 in gastric cancer and correlation with tumor invasion and prognosis. *World J Gastroenterol* 2014; **20**: 6906-6911 [PMID: 24944481 DOI: 10.3748/wjg.v20.i22.6906]

108   **Fu Z**, Qian F, Yang X, Jiang H, Chen Y, Liu S. Circulating miR-222 in plasma and its potential diagnostic and prognostic value in gastric cancer. *Med Oncol* 2014; **31**: 164 [PMID: 25129310 DOI: 10.1007/s12032-014-0164-8]

109   **Zhou X**, Ji G, Chen H, Jin W, Yin C, Zhang G. Clinical role of circulating miR-223 as a novel biomarker in early diagnosis of cancer patients. *Int J Clin Exp Med* 2015; **8**: 16890-16898 [PMID: 26629240]

110   **Fassan M**, Saraggi D, Balsamo L, Realdon S, Scarpa M, Castoro C, Coati I, Salmaso R, Farinati F, Guzzardo V, Arcidiacono D, Munari G, Gasparini P, Veronese N, Luchini C, Valeri N, Rugge M. Early miR-223 Upregulation in Gastroesophageal Carcinogenesis. *Am J Clin Pathol* 2017; **147**: 301-308 [PMID: 28395057 DOI: 10.1093/ajcp/aqx004]

111   **Zhuang K**, Han K, Tang H, Yin X, Zhang J, Zhang X, Zhang L. Up-Regulation of Plasma miR-23b is Associated with Poor Prognosis of Gastric Cancer. *Med Sci Monit* 2016; **22**: 356-361 [PMID: 26835790 DOI: 10.12659/MSM.895428]

112   **Li BS**, Zuo QF, Zhao YL, Xiao B, Zhuang Y, Mao XH, Wu C, Yang SM, Zeng H, Zou QM, Guo G. MicroRNA-25 promotes gastric cancer migration, invasion and proliferation by directly targeting transducer of ERBB2, 1 and correlates with poor survival. *Oncogene* 2015; **34**: 2556-2565 [PMID: 25043310 DOI: 10.1038/onc.2014.214]

113   **Yan C**, Yu J, Liu Y, Kang W, Ma Z, Zhou L. MiR-32 promotes gastric carcinoma tumorigenesis by targeting Kruppel-like factor 4. *Biochem Biophys Res Commun* 2015; **467**: 913-920 [PMID: 26471298 DOI: 10.1016/j.bbrc.2015.10.044]

114   **Sandoval-Bórquez A**, Polakovicova I, Carrasco-Véliz N, Lobos-González L, Riquelme I, Carrasco-Avino G, Bizama C, Norero E, Owen GI, Roa JC, Corvalán AH. MicroRNA-335-5p is a potential suppressor of metastasis and invasion in gastric cancer. *Clin Epigenetics* 2017; **9**: 114 [PMID: 29075357 DOI: 10.1186/s13148-017-0413-8]

115   **Lo SS**, Hung PS, Chen JH, Tu HF, Fang WL, Chen CY, Chen WT, Gong NR, Wu CW. Overexpression of miR-370 and downregulation of its novel target TGFβ-R II contribute to the progression of gastric carcinoma. *Oncogene* 2012; **31**: 226-237 [PMID: 21666718 DOI: 10.1038/onc.2011.226]

116   **Zhang WH**, Gui JH, Wang CZ, Chang Q, Xu SP, Cai CH, Li YN, Tian YP, Yan L, Wu B. The identification of miR-375 as a potential biomarker in distal gastric adenocarcinoma. *Oncol Res* 2012; **20**: 139-147 [PMID: 23461060 DOI: 10.3727/096504012X1352222723 2156]

117   **Hung PS**, Chen CY, Chen WT, Kuo CY, Fang WL, Huang KH, Chiu PC, Lo SS. miR-376c promotes carcinogenesis and serves as a plasma marker for gastric carcinoma. *PLoS One* 2017; **12**: e0177346 [PMID: 28486502 DOI: 10.1371/journal.pone.0177346]

118   **Zhou H**, Xiao B, Zhou F, Deng H, Zhang X, Lou Y, Gong Z, Du C, Guo J. MiR-421 is a functional marker of circulating tumor cells in gastric cancer patients. *Biomarkers* 2012; **17**: 104-110 [PMID: 22263628 DOI: 10.3109/1354750X.2011.614961]

119   **Zhao G**, Xu L, Hui L, Zhao J. Level of circulated microRNA-421 in gastric carcinoma and related mechanisms. *Int J Clin Exp Pathol* 2015; **8**: 14252-14256 [PMID: 26823741]

120   **Wu J**, Li G, Yao Y, Wang Z, Sun W, Wang J. MicroRNA-421 is a new potential diagnosis biomarker with higher sensitivity and specificity than carcinoembryonic antigen and cancer antigen 125 in gastric cancer. *Biomarkers* 2015; **20**: 58-63 [PMID: 25510566 DOI: 10.3109/1354750X.2014.992812]

121   **Shah AA**, Leidinger P, Backes C, Keller A, Karpinski P, Sasiadek MM, Blin N, Meese E. A set of specific miRNAs is connected with murine and human gastric cancer. *Genes Chromosomes Cancer* 2013; **52**: 237-249 [PMID: 23124995 DOI: 10.1002/gcc.22024]

122   **Li SC**, Liao YL, Ho MR, Tsai KW, Lai CH, Lin WC. miRNA arm selection and isomiR distribution in gastric cancer. *BMC Genomics* 2012; **13** Suppl 1: S13 [PMID: 22369582 DOI: 10.1186/1471-2164-13-S1-S13]

123   **Telonis AG**, Rigoutsos I. Race Disparities in the Contribution of miRNA Isoforms and tRNA-Derived Fragments to Triple-Negative Breast Cancer. *Cancer Res* 2018; **78**: 1140-1154 [PMID: 29229607 DOI: 10.1158/0008-5472.CAN-17-1947]

124   **Gyvyte U**, Juzenas S, Salteniene V, Kupcinskas J, Poskiene L, Kucinskas L, Jarmalaite S, Stuopelyte K, Steponaitiene R, Hemmrich-Stanisak G, Hübenthal M, Link A, Franke S, Franke A, Pangonyte D, Lesauskaite V, Kupcinskas L, Skieceviciene J. MiRNA profiling of gastrointestinal stromal tumors by next-generation sequencing. *Oncotarget* 2017; **8**: 37225-37238 [PMID: 28402935 DOI: 10.18632/oncotarget.16664]

125   **Lawson DA**, Bhakta NR, Kessenbrock K, Prummel KD, Yu Y, Takai K, Zhou A, Eyob H, Balakrishnan S, Wang CY, Yaswen P, Goga A, Werb Z. Single-cell analysis reveals a stem-cell program in human metastatic breast cancer cells. *Nature* 2015; **526**: 131-135 [PMID: 26416748 DOI: 10.1038/nature15260]

Nutritional issues in patients with obesity and cirrhosis

Luigi Schiavo, Luca Busetto, Manuela Cesaretti, Shira Zelber-Sagi, Liat Deutsch, Antonio Iannelli

Luigi Schiavo, Department of Translational Medical Science, University of Campania "Luigi Vanvitelli", Naples 80131, Italy

Luigi Schiavo, IX Division of General Surgery, Vascular Surgery and Applied Biotechnology, Naples University Policlinic, Naples 80131, Italy

Luca Busetto, Department of Medicine, University of Padua, Padua 35128, Italy

Luca Busetto, Center for the Study and the Integrated Management of Obesity, University Hospital of Padua, Padua 35128, Italy

Manuela Cesaretti, Department of HPB Surgery and Liver Transplantation, Hôpital Beaujon, AP-HP, Clichy 92110, France

Manuela Cesaretti, Department of Nanophysics, Italian Institute of Technology, Genova 16163, Italy

Shira Zelber-Sagi, School of Public Health, University of Haifa, Haifa 3498838, Israel

Shira Zelber-Sagi, Liat Deutsch, Department of Gastroenterology and Liver disease, Tel Aviv Medical Center, 62431, Tel-Aviv 62431, Israel

Liat Deutsch, The Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv 62431, Israel

Antonio Iannelli, Digestive Unit, Archet 2 Hospital, University Hospital of Nice, F-06202, Nice, France; Inserm, U1065, Team 8 "Hepatic complications of obesity", Nice F-06204, France

Antonio Iannelli, University of Nice Sophia-Antipolis, Nice F-06107, France

ORCID number: Luigi Schiavo (0000-0003-3639-6847); Luca Busetto (0000-0003-4883-8980); Manuela Cesaretti (0000-0001-6419-1481); Shira Zelber-Sagi (0000-0002-1324-7497); Liat Deutsch (0000-0001-5022-4318); Antonio Iannelli (0000-0002-1611-071X).

Abstract

Obesity and metabolic syndrome are considered as responsible for a condition known as the non-alcoholic fatty liver disease that goes from simple accumulation of triglycerides to hepatic inflammation and may progress to cirrhosis. Patients with obesity also have an increased risk of primary liver malignancies and increased body mass index is a predictor of decompensation of liver cirrhosis. Sarcopenic obesity confers a risk of physical impairment and disability that is significantly higher than the risk induced by each of the two conditions alone as it has been shown to be an independent risk factor for chronic

liver disease in patients with obesity and a prognostic negative marker for the evolution of liver cirrhosis and the results of liver transplantation. Cirrhotic patients with obesity are at high risk for depletion of various fat-soluble, water-soluble vitamins and trace elements and should be supplemented appropriately. Diet, physical activity and protein intake should be carefully monitored in these fragile patients according to recent recommendations. Bariatric surgery is sporadically used in patients with morbid obesity and cirrhosis also in the setting of liver transplantation. The risk of sarcopenia, micronutrient status, and the recommended supplementation in patients with obesity and cirrhosis are discussed in this review. Furthermore, the indications and contraindications of bariatric surgery-induced weight loss in the cirrhotic patient with obesity are discussed.

Key words: Obesity; Cirrhosis; Sarcopenia; Malnutrition; Bariatric surgery

Core tip: Obesity is a frequent cause of chronic liver disease that can progress to cirrhosis. Cirrhotic patients with obesity frequently have alterations in specific aspects of nutritional status, such as poor protein intake and micronutrient deficiencies. Diet, physical activity and protein intake should be carefully monitored. Bariatric surgery may be an option in the management of patients with morbid obesity and cirrhosis also in the setting of liver transplantation but scientific evidence is still scarce.

INTRODUCTION

Liver cirrhosis and obesity: Definitions and epidemiology

Obesity is associated with many adverse consequences for health and is closely related to insulin resistance, dyslipidemia, and hypertension. Non-alcoholic fatty liver disease (NAFLD) represents the hepatic manifestation of metabolic syndrome and is intimately related to the chronic intrahepatic inflammation that, in turn, is linked to adiposity and insulin resistance. Defined as a fatty infiltration of the liver exceeding 5% at histology in the absence of previous or ongoing significant alcohol consumption[1] and/or drug and/or virus infection, NAFLD includes a wide spectrum of histopathological alterations ranging from simple steatosis (Figure 1A) to non-alcoholic steatohepatitis (NASH) (Figure 1B) and cirrhosis (Figure 1C) with end-stage liver disease. Furthermore, NAFLD has been also shown to increase the risk of primary liver malignancies such as hepatocellular carcinoma

(HCC)[2-4]. However, the vast majority of patients with NAFLD will not progress as only a minority of those with NASH (3%-5%)[5] are at a high risk of developing chronic liver disease complications. In patients with NASH, 11% develop cirrhosis and approximately 40% of patients die within 15 years from any cause (of which 7.3% are due to liver-related complications, especially in those with advanced fibrosis or cirrhosis)[6]. Obesity and the chronic inflammation associated with it also have a negative effect on health. Excessive adipose tissue leads to an increased production of adipokines (such as IL-6, TNFa, monocyte chemoattracting protein-1, and plasminogen activator inhibitor-1) with proinflammatory, pro-fibrogenic, pro-angiogenic, and pro-oxidant effects on several tissues. Few data exist regarding the epidemiological and clinical impact of obesity in patients with pre-existent liver disease, however, obesity is considered an independent risk factor for the presence of severe fibrosis, fibrosis progression, and cirrhosis[7,8]. Several population-based studies have identified obesity as an independent risk factor for alcohol-induced liver damage. Indeed, ethanol influences the adipose tissue production of hormones and cytokines, and excess adiposity induces an exacerbation of the proinflammatory state. However, it is still unclear whether the hepatotoxic consequences of obesity and ethanol ingestion are additive or synergistic. The DIONYSOS study clearly showed a synergistic effect between alcohol consumption and elevated body mass index (BMI) on hepatic steatosis in a large cohort of subjects in Northern Italy[9]. The prevalence of hepatic steatosis determined by ultrasonography was increased to 46% in subjects with a daily intake of > 60 g of alcohol and to 76% in patients with obesity compared to lean controls who only revealed hepatic steatosis in 16% of cases. Moreover, in individuals with obesity drinking > 60 g of alcohol per day, steatosis was found at an even higher level of 95% and the relative risk of cirrhosis increased more than six-fold in women with obesity and alcohol consumption (> 150 g/wk) *vs* normal weight and drinking < 70 g/wk women. Ekstedt *et al*[10] found an accelerated progression of fibrosis in patients with NAFLD who drank moderate amounts of alcohol (up to 140 g/wk). Liu *et al*[11] analyzed more than 1 million middle-aged women in the UK and reported that in women with obesity who drank > 150 g of alcohol/week, the relative risk of cirrhosis increased more than six fold.

In patients with chronic hepatitis C virus (HCV)-related liver disease and severe liver fibrosis or compensated cirrhosis, up to 43% had obesity, and 32% were overweight. Each quartile increase in BMI is associated with a 14% increase in the risk of clinical events in the follow-up (worsening of liver fibrosis or decompensation of cirrhosis over 3.5 years of follow-up)[12]. Among obesity-related variables, the severity of insulin resistance and the histological grade of steatosis appeared as the factors more tightly associated with the progression of liver disease. Contrarily, weight loss is indeed beneficial in patients with advanced chronic liver disease, reducing the progression of fibrosis and cirrhosis[13,14].
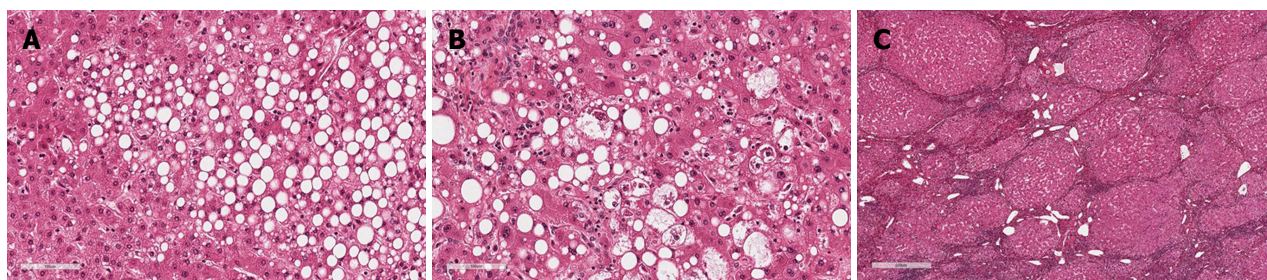
Figure 1 Non-alcoholic fatty liver disease and hepatic histopathological alterations. NAFLD includes a wide spectrum of histopathological alterations ranging from simple steatosis (A) to non-alcoholic steatohepatitis (B) and cirrhosis (C). NAFLD: Non-alcoholic fatty liver disease.

Berzigotti *et al*[13,14] demonstrate that increased BMI is a strong predictor of decompensation in patients with compensated cirrhosis of various etiologies, independent of other previously described predictors such as albumin and portal hypertension. More specifically, clinical decompensation of cirrhosis (ascites, encephalopathy, or jaundice) developed in 14% of patients with normal weight, in 31% of overweight patients, and in 43% of patients with obesity. Obesity also negatively impacts portal hypertension. Indeed, when comparing the results after a 1-year course of timolol or a placebo, portal hypertension was reduced only in patients who were of normal weight or overweight, whereas patients with obesity showed a significant increase.

Even in patients with end-stage liver disease and, therefore, awaiting liver transplantation (LT), obesity could worsen the prognosis. Recent data suggest that in these patients, obesity increases substantially (HR = 13.1), independently and significantly (P = 0.016) the risk of portal vein thrombosis[15], which may render the transplantation technically more difficult and increase the risk of portal thrombosis recurrence. Indeed, obesity is considered as a risk factor for venous thromboembolism as well as thrombosis of the hepatic artery[16]. The proinflammatory, prothrombotic, and hypofibrinolytic milieu patients with obesity may be responsible for the local thrombophilia that favors portal vein thrombosis. Concerning perioperative LT complications, operative time, blood product usage, length of stay in the intensive care unit, infectious complications, and biliary complications requiring intervention have been shown to be higher in recipients with obesity[17]. Despite the increased technical operative challenges and medical complexities associated with recipients with obesity, morbid obesity in itself should not be an absolute contraindication to LT as these patients have reasonable long-term outcomes. Although the complex interplay between obesity and cirrhosis is far from elucidated, undoubtedly alcohol, viral hepatitis, and obesity appear to be a dangerous combination. General impact of obesity on liver pathophysiology are summarized in Table 1.

RISK OF SARCOPENIA IN PATIENTS WITH OBESITY AND CIRRHOSIS

Sarcopenia is a syndrome characterized by progressive and generalized loss of skeletal muscle mass and strength with a risk of adverse outcomes such as physical disability, poor quality of life, and death[18]. The profound negative clinical impact of low skeletal muscle mass and function was originally described in the elderly, but its role is now unequivocally emerging in many chronic progressive diseases, such as chronic kidney disease, chronic heart failure, chronic pulmonary insufficiency, and type 2 diabetes[18]. Recent evidence suggests that low muscle mass and function may have a similar negative impact in obesity despite the potential difficulties in identifying and defining muscle changes within the obese phenotype[19]. Whatever the definition, the coexistence of sarcopenia and obesity in the same patient, now indicated as "sarcopenic obesity", confers a risk of physical impairment and disability that is significantly higher than the risk induced by each of the two conditions alone[19-21]. The precise determination of body muscle and body mass fat requires the use of dual energy X-ray absorptiometry (DXA), but an estimation can be obtained at the clinical level with the use of bioimpedance analysis (BIA) or anthropometric measurements[18]. In this section, the role of sarcopenic obesity as a prognostic factor in patients with liver chronic disease will be briefly elucidated. The problem will be analyzed according to two different perspectives. First, the possible role played by sarcopenia as an independent risk factor for the occurrence of liver diseases in patients with obesity will be discussed and, second, the significance of sarcopenia as a negative prognostic marker for disease progression and death in patients with obesity and advanced liver disease will be addressed.

Sarcopenia as an independent risk factor for chronic liver disease in patients with obesity

As already mentioned above, NAFLD is today the most common liver disorder in Western countries and it is becoming the leading cause of chronic liver disease and cirrhosis[22]. Visceral obesity and related metabolic disorders, insulin resistance, in particular, are considered the most relevant risk factors for NAFLD occurrence and progression[22]. Recent evidence suggests, however, that the prevalence of NAFLD and its severity could be independently and negatively affected also by the coexistence of sarcopenia in the clinical picture, with sarcopenic obesity being both an independent risk factor for NAFLD and a marker for its progression to the more

Table 1 Impact of obesity on liver pathophysiology

Pathologies	Obesity
Hepatic steatosis[9]	Increased[1]
Cirrhosis[11]	Increased[1]
Hepatoxicity[9]	Increased[1]
Liver primary tumors (as hepatocarcinoma)[2-4]	Increased[2]
Chronic hepatitis C progression[12]	Increased
Decompensation of cirrhotic patients[13,14]	Increased
Portal hypertension[13,14]	Increased
[3]Risk of post-operative complications after LT[15-17]	Increased

[1]Mostly if associated to alcohol ingestion; [2]Mostly if associated to non-alcoholic fatty liver disease (NAFLD); [3]Thromboembolism, infectious and biliary complications. LT: Liver transplantation.
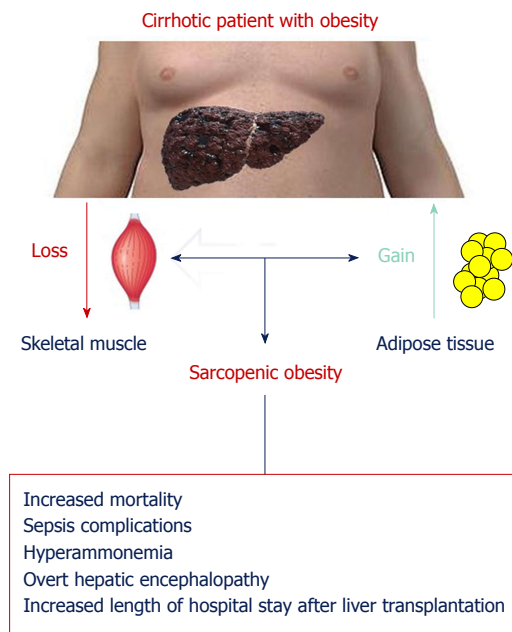

Figure 2 Sarcopenia as a prognostic negative marker in the cirrhotic patient with obesity. Cirrhotic patients with obesity frequently have a combined loss of skeletal muscle and gain of adipose tissue, culminating in the condition known as "*sarcopenic obesity*". Sarcopenia in cirrhotic patients has been associated with increased mortality, sepsis complications, hyperammonemia, overt hepatic encephalopathy, and an increased length of hospital stay after liver transplantation.

advanced stages and cirrhosis[23].

Significant information about the role of sarcopenia in chronic liver disease was produced in an analysis of data from the Korea National Health and Nutrition Examination Survey (KNHANES). KNHANES includes a nationwide cross-sectional cohort with a representative sample of the Korean population and is conducted annually to assess health and nutritional status. Hong *et al*[24], by analyzing 452 apparently healthy adults in the Korean Obesity Sarcopenic Study, demonstrated a higher risk of NAFLD in individuals with lower muscle mass compared to a control group. Individuals with sarcopenia had more body fat mass, more components of the metabolic syndrome, and higher levels of low-grade systemic inflammation compared with those of people with normal muscle mass. Moreover, the association between sarcopenia retained its significance also after adjustment for all these potential confounding factors[24]. More recently, Lee *et al*[25] tested the association between sarcopenia and progression of NALFD by analyzing the degree of liver fibrosis in the KNHANES population. NAFLD was identified in 2761 (28.5%) of 9676 subjects and sarcopenia was identified in 337 of the subjects with NAFLD (12.2%). In subjects with NAFLD, sarcopenia was significantly and independently associated with a higher level of liver fibrosis. The authors concluded that sarcopenia was associated with significant liver fibrosis in subjects with NAFLD, and this association was independent of obesity and insulin resistance[25].

The independent and additive role of reduced skeletal muscle mass and increased visceral fat mass in increasing the risk of the onset of NAFLD, and in serving as important factors involved in the progression from simple steatosis to liver fibrosis, has been recently confirmed in an independent Japanese sample[26]. Taking into consideration the limitation of these studies and the caveats in extrapolating these data to the Caucasian population, the association of sarcopenia with visceral obesity and insulin resistance is now considered an important risk factor for NAFLD, which further accelerates its progression to more advanced life-threatening stages.

Sarcopenia as a prognostic negative marker in the cirrhotic patient with obesity

As shown in Figure 2, cirrhotic patients with obesity frequently have a combined loss of skeletal muscle and gain of adipose tissue, culminating in the condition known as "*sarcopenic obesity*." Sarcopenia in cirrhotic patients has been associated with increased mortality, sepsis complications, hyperammonemia, overt hepatic encephalopathy (HE), and an increased length of hospital stay after LT[27]. The prognostic impact of the coexistence of sarcopenia and obesity in patients with chronic liver disease has been analyzed recently in a few studies. Montano-Loza *et al*[28] evaluated the frequencies of sarcopenia and sarcopenic obesity in a cohort of 457 cirrhotic patients evaluated for LT, aiming to establish the impact of these muscular abnormalities on the prognosis of cirrhotic patients. In this sample, sarcopenia was present in 43% and sarcopenic obesity in 20% of patients. Both patients with sarcopenia and with sarcopenic obesity had worse median survival than patients without muscular abnormalities[28]. Hara *et al*[29] evaluated the independent prognostic impact of skeletal muscle mass and visceral fat accumulation in 161 patients with cirrhosis. Patients with sarcopenia or sarcopenic obesity both had a poor prognosis, and this difference was pronounced in the subset of patients classified as Child-Pugh class A. However, the group with the worst prognosis was represented by patients having sarcopenic obesity[29]. This latter observation seems to confirm that the coexistence of visceral obesity and sarcopenia could be considered the worst clinical

situation for a patient with cirrhosis. Muscle wasting and fatty muscle infiltration in cirrhotic patients are part of the frailty complex present in these patients, characterized as decreased reserve and resistance to stressors, resulting from cumulative declines across multiple physiologic systems and predisposition to poor outcomes[28].

The prognostic negative role of sarcopenia and obesity could have an impact also in LT. Sarcopenia and sarcopenic obesity are seen in a significant number of patients with cirrhosis undergoing liver transplant evaluation, particularly in patients with NASH[30]. Pre-transplant sarcopenia is widely recognized as associated with short-term survival after living donor LT[31]. The question of whether the coexistence of obesity and sarcopenia could impose additional risk after LT over that imposed by sarcopenia alone is still debated. Hammad *et al*[32] evaluated 200 patients undergoing adult-to-adult living donor LT and classified them into four subgroups: sarcopenic overweight, sarcopenic non-overweight, non-sarcopenic overweight, and non-sarcopenic non-overweight. Sarcopenic patients had a higher incidence of postoperative bacteremia and major postoperative complications, and poorer overall post-transplant survival than non-sarcopenic patients. Overweight recipients had a significantly higher overall survival rate than non-overweight patients. In contrast, sarcopenic non-overweight subjects had a higher incidence of postoperative bacteremia, and major postoperative complications compared with the sarcopenic overweight subgroup and possessed the poorest overall survival among the four recipient subgroups. Thus, this study indicates that a preoperative sarcopenic overweight status does not confer additional significant morbidity or mortality risks than the stand-alone sarcopenia in living donor LT. The study was however mostly confined to patients who were overweight and does not address specifically the impact of obesity itself[32]. On the other hand, obesity presents important medical and surgical challenges during and after a liver transplant. Specifically, obesity is associated with an increased incidence of wound infections, wound dehiscence, biliary complications, and overall infection[33].

Sarcopenic obesity could represent an important clinical problem also for long-term survival after LT. Sarcopenia tends to persist after transplantation[30,31], possibly as the consequence of insufficient physical exercise or use of immunosuppressive agents that impair skeletal muscle growth and protein accretion[30], whereas body fat normally increased as a result of better nutrition and the regression of the hypermetabolic state related to the pre-transplant chronic disease state[34]. This double phenomenon paves the way to sarcopenic obesity. Schütz *et al*[35] analyzed body composition in patients after liver and kidney transplantation and patients with liver cirrhosis or on chronic hemodialysis and demonstrated that, despite excellent graft function, many long-term liver or kidney transplant survivors exhibit a phenotype of sarcopenic obesity with increased fat but low muscle mass[35]. Choudhary *et al*[36] evaluated

82 living donor liver transplant recipients with at least 12 mo of follow-up. Post-transplant sarcopenic obesity was present in 88%, and metabolic syndrome was present in 52% of recipients with no significant difference among liver failure etiologies. Patients with sarcopenic obesity had a significantly higher BMI and significantly higher prevalence of metabolic syndrome when compared to patients without sarcopenic obesity[36]. Thus, the weight gain frequently observed after LT could contribute to the creation of a novel group of patients with sarcopenic obesity and associated metabolic derangements.

MICRONUTRIENT STATUS AND RECOMMENDED SUPPLEMENTATION IN PATIENTS WITH OBESITY AND CIRRHOSIS

Micronutrient deficiencies of fat-soluble and water-soluble vitamins as well as minerals are highly common in end-stage liver disease. Therefore, a periodical nutritional and clinical assessment should be performed to test micronutrient serum levels, estimating adequate nutritional intake and paying attention to clinical signs of vitamins and minerals deficiencies. Fat-soluble vitamin (A, D, E, and K) deficiencies and other free radical scavengers, such as carotenoids are likely to develop in liver disease due to reduced oral intake, malabsorption, and/or hepatic effects involving the reduced synthesis of carrier and transfer proteins, cholestasis and bacterial overgrowth[37,38].

Deficiencies in water-soluble vitamins, leading to neuropsychiatric symptoms, may stem from diminished hepatic storage, inadequate diet, alcoholism, medications, and chronic renal failure[37]. The most prevalent and significant deficiencies are discussed below, beginning with vitamin D deficiency, which is almost universal and also related with obesity.

Vitamin D deficiency (VDD, < 50 nmol/L; < 20 ng/mL) is a worldwide pandemic, reported in more than half of the general adult population[39-42]. Specifically, VDD is widespread among patients with chronic liver disease, with prevalence described between 70%-90%[40,43-49], while the prevalence of vitamin D insufficiency (< 75 nmol/L; < 30 ng/mL) is almost universal[49]. For example, among 202 adults before LT, 84% were 25(OH)D deficient and 77% had low 1,25(OH)2D, whereas 3 mo following transplantation, both increased significantly but to a lesser extent for 1,25(OH)2D[50]. Moreover, the severity of VDD is positively correlated with the severity of liver disease[44-47]. In cirrhotic patients, significant correlations were found between vitamin D levels and the degree of liver dysfunction, whether expressed as Child-Pugh stages A-C, MELD score, or liver stiffness evaluated by transient elastography[44,45]. In epidemiologic studies, VDD has been demonstrated to be a predictor of liver disease morbidity and mortality. In a Danish population-based sample of over 2500 middle-

aged subjects followed prospectively for a median follow-up period of 16.5 years, lower serum 25(OH)D levels were independently associated with higher incidence of fatal and non-fatal liver disease[51]. Similarly, in another prospective study with 22 years follow-up[52], those with a higher serum 25(OH) vitamin D had lower chronic liver disease mortality. Among cirrhotic patients, VDD was found to be an independent risk factor for mortality[43,45]. With the increasing prevalence of obesity and NAFLD-related cirrhosis, attention should be paid to the cirrhotic patient with obesity. An association between VDD and obesity has also been suggested. Since vitamin D is a lipophilic molecule it is selectively deposited in subcutaneous and visceral adipose tissue. This sequestration leads to a reduction of its availability for hydroxylation[40,41,53,54]. In addition, an independent association was also observed between VDD and insulin resistance[55,56], the metabolic syndrome as a whole[57], and type 2 diabetes or prediabetes, after adjusting for multiple confounders including BMI[55,56,58,59]. In contrast, some studies failed to show such associations once adiposity is adjusted for[60,61] or any significant association between markers of insulin resistance and 25(OH)D serum levels[62,63]. NAFLD by itself has been suggested to be associated with VDD, but the independence of this association from body fat mass is controversial. The association between a diagnosis of NAFLD and lower serum 25(OH)D levels compared to healthy controls was demonstrated in several studies[64-67], however, NAFLD was biopsy-proven in only two of the studies[64,65]. In addition, significantly higher levels of 25(OH)D were observed among patients with steatosis compared to patients with NASH[64,65]. In partial agreement, another study, describing a sub-analysis of the NASH Clinical Research Network (CRN) cohort, found that VDD was independently associated with NASH and the presence of fibrosis, but not with the degree of steatosis[68]. However, while most studies adjusted for BMI or waist circumference, a study carefully adjusting for adiposity (evaluated by dual-energy X-ray absorptiometry), suggests that there is no relationship between vitamin D levels and the amount of liver fat (by Magnetic Resonance Spectroscopy and liver biopsy) or the severity of NASH[69].

To summarize, a few possible mechanisms could explain VDD among cirrhotic patients with obesity: (1) NAFLD as the undelaying cause of cirrhosis; (2) Sedentary lifestyle or long standing chronic illness leading to reduced exposure to sunlight; (3) Consumption of high-caloric "empty" foods, low in mineral and vitamin content; (4) Increased fat mass which enables sequestration of vitamin D in the adipose tissue compartment thus lowering plasma concentration; and (5) Hepatocellular dysfunction in chronic liver disease which may disturb the formation of the active metabolite of vitamin D as well as vitamin D-binding proteins.

Due to the high prevalence of VDD among cirrhotic patients, especially those with advanced disease, NAFLD, or cholestatic liver diseases[70], it is reasonable to assess serum 25(OH)D levels[71]. The main sources of vitamin D are sunlight exposure, food naturally enriched or artificially fortified with vitamin D, and oral supplements. The adequate sunlight exposure is 5-15 min during the daytime (10:00 AM-15:00 PM) on a daily basis[40]. However, after exposing healthy individuals with obesity (BMI > 30 kg/m^2) and matched lean control subjects (BMI < 25 kg/m^2) to whole-body ultraviolet radiation, the subjects with obesity showed a 57% lower increase in circulating concentrations of vitamin D3, 24 h after irradiation[53]. Vitamin D3 can be found abundantly in egg yolk and oily fish (e.g. salmon, mackerel, tuna, sardines) and vitamin D2 mainly in mushrooms[40,49]. Industrial products such as bread, margarine, cereals, or milk may be fortified with either vitamin D2 or vitamin D3. Oral supplements may contain vitamin D2 or D3 depending on the manufacturer and brand[40,49]. However, most studies that have evaluated NAFLD and high-dose vitamin D supplementation, failed to show improvement in liver fat, NASH histology, or liver enzymes[72,73]. Oral high-dose vitamin D supplementation administrated to subjects with NAFLD and type-2 diabetes for 24 wk, did not lead to significant change in hepatic steatosis, HOMA-IR, or liver enzymes[74]. Similarly, in a pilot prospective study, administration of high-dose oral vitamin D3 supplementation (25000 IU/wk) for 24 wk to 12 non-cirrhotic patients with biopsy-proven NASH resulted in no significant impact on liver histology, liver enzymes, insulin resistance, or adipocytokine profile[73]. Conversely, some studies, not specific for NAFLD but mostly pre-diabetic patients, demonstrated improvement in insulin sensitivity with vitamin D supplementation[75-77]. Findings are also conflicting with regard to the effect of vitamin D supplementation in the case of advanced liver disease. In a cohort of advanced decompensated cirrhotic patients (Child-Pugh C, score of $\geq$ 10) randomly assigned to vitamin D treatment or a control receiving standard care and followed for 6 mo, the survival, as well as Child-Pugh and MELD scores, were comparable between the groups[48]. Nevertheless, supplementation of high-dose oral vitamin D resulted in several clinical improvements such as an anti-depressant effect among patients with chronic liver disease (mainly women)[78], and 60% risk reduction for acute rejection among patients after LT[79]. In a Cochrane review of vitamin D supplementation for chronic liver diseases in adults, including 15 randomized clinical trials (RCT) with 1034 participants[80], vitamin D supplementation had no beneficial or harmful effects on all-cause mortality (OR = 0.69; 95%CI: 0.09 to 5.40), and the authors concluded that there is no convincing evidence that vitamin D supplementation has a therapeutic impact in chronic liver disease.

There are no guidelines or clear recommendations regarding vitamin D supplementation for cirrhotic patients with obesity. One authoritative paper[49] recommended vitamin D status assessment in all patients with chronic liver disease and initiation of supplementation with 1000-4000 IU/d of vitamin D3 when 25(OH)D levels are under < 50 nmol/L or < 20 ng/mL. The

guidelines of a consensus workshop supported by the British Association for the Study of the Liver and the British Liver Trust, recommends supplementation of 1 g calcium and 800 IU/D vitamin D to patients with chronic liver disease (especially patients with cirrhosis or severe cholestasis) as a general preventive treatment for osteoporosis along with adequate nutrition monitoring[81]. The European Association for the Study of the Liver (EASL) recommendation includes supplementation with calcium (1000-1200 mg/d) and vitamin D (400-800 IU/d) in all patients with cholestatic liver disease for bone loss reduction, although the evidence to support this is low[70]. Interestingly, in a small study of NAFLD patients, daily supplementation with 2000 IU cholecalciferol for 6 mo did not correct hypovitaminosis D in the majority of patients with NASH compared to those with steatosis, thus a higher dose may be needed in NASH or NASH-cirrhosis patients but this finding needs further confirmation[82].

Vitamin E (tocopherol) is an inexpensive and well-tolerated molecule with antioxidant properties. In vitro and animal studies have shown that vitamin E ameliorates liver necrosis and fibrosis[83], and prevents hepatic stellate cell activation[84]. Relevantly for patients with obesity and liver disease, high-dose (800 IU/d) long-term (24 mo) vitamin E treatment among nondiabetic NASH patients led to improvement in NASH versus placebo but not fibrosis. However, cirrhotic patients were excluded from this RCT[85], and thus vitamin E cannot be recommended for NASH-cirrhosis or cryptogenic cirrhosis patients[86]. Vitamin E status, expressed as serum vitamin E/total serum cholesterol ratio, is lower among patients with primary biliary cholangitis and primary sclerosing cholangitis compared to patients with either cryptogenic or alcoholic cirrhosis, probably due to reduced gastrointestinal absorption[87]. Indeed, a reduced concentration of vitamin E and low vitamin E/total cholesterol ratio has been demonstrated in 44% and 64% of patients with primary biliary cholangitis and 32% and 43% of patients with other chronic cholestatic liver diseases, respectively. Serum vitamin E concentration and vitamin E/total cholesterol ratio were restored to normal by oral or intramuscular supplements of the vitamin, but not in patients with severe deficiency of vitamin E (less than 5 μmol/L and less than 1 μmol/mmol total cholesterol)[88]. Generally, carotenoids, as well as tocopherols, are major natural protective agents against free radical-mediated liver damage. Interestingly, it has been demonstrated that patients with cirrhosis (of mixed etiologies) have extremely low hepatic levels of carotenoids and tocopherols, compared with controls, even in the presence of normal serum levels[89]. Similarly, retinol, a-tocopherol, and carotenoid plasma levels in patients with chronic cholestatic liver disease (primary biliary cholangitis and primary sclerosing cholangitis) were demonstrated to be significantly lower compared to the general population, despite comparable nutritional intake, suggesting a role for the malabsorption of fat-soluble vitamins[38]. Clinical manifestations of vitamin E deficiency may include increased platelet aggre-gation, decreased red blood cell survival, hemolytic anemia, decreased serum creatinine with creatinuria, and neuronal degeneration[37], but these are not common[38,87,88].

Zinc is the second most prevalent trace element in the body, playing a central role in several metabolic, anti-inflammatory and immune response pathways, with more than 300 enzymes having zinc ions within their catalytic domains[90]. Dietary zinc is prevalent in foods which are rich in protein (*i.e.* red meat, lamb, pork, oysters, *etc.*) and its absorption can be inhibited by high consumption of foods rich in phytate, certain dietary fibers, and calcium[91,92]. The recommended dietary allowance (RDA) of zinc is 8 mg/d for women and 13 mg/d for men over 19 years old[93]. The liver is the main organ of zinc metabolism, however, in chronic liver disease, metabolism is altered by inadequate dietary intake, protein and amino acid metabolism alterations, diminished hepatic extraction, portosystemic shunts, impaired absorption (mostly in advanced liver disease), and an increase in various inflammatory cytokines[90,94]. Moreover, cirrhosis is a catabolic state resulting in muscle catabolism leading to increased zinc loss in the urine. This urinary loss of zinc is aggravated by the prevalent use of diuretics in order to treat edema and ascites[95]. Zinc deficiency has many clinical implications in cirrhotic patients. One of the major complications in cirrhotic patients is HE, presented in 30%-40% of the patients as overt encephalopathy (OHE) and in 60%-80% as mild cognitive dysfunction (minimal hepatic encephalopathy, MHE)[96]. Zinc deficiency results in impaired nitrogen metabolism due to the reduced enzymatic activity of urea cycle enzymes and decreased muscle glutamine synthesis. Furthermore, zinc levels were found to be negatively correlated with ammonia serum levels[97,98]. These impaired metabolic pathways can be corrected by supplementing zinc. In a small preliminary double-blind, placebo-controlled trial among patients with cirrhosis, hyperammonemia, and hypozincemia, zinc acetate preparation at a dose of 150 mg/d for 3 mo decreased blood ammonia levels[99]. Indeed, Marchesini *et al*[100] demonstrated in patients with advanced cirrhosis, that long-term treatment with zinc supplementation accelerated and improved hepatic conversion of amino acids to urea compared with matched controls receiving standard treatment. This biochemical improvement was associated with a clinical improvement measured by the performance in psychometric tests and Child-Pugh score. Similar results were observed in an RCT comparing zinc supplementation on top of standard treatment *vs*. standard treatment alone (protein-restricted diet, branched-chain amino acids, and lactulose) for 6 mo. Zinc supplementation improved the physical component scale and neuropsychological tests and decreased HE grade, Child-Pugh score and blood ammonia levels[101]. A significant improvement in neuropsychological tests and Child-Pugh score along with ammonia was also reported in patients with only MHE after 3 mo of lactu-lose, antioxidant and zinc therapy *vs* lactulose therapy

alone[102]. Additionally, zinc is a crucial co-factor in the process of wound healing since it activates the synthesis of collagen and metabolism of nucleic acids. Thus, zinc is an essential factor in the restoration of liver parenchyma after liver injury or resection and is required in large amounts over a short period of time[90,103]. In terms of clinical signs for zinc deficiency, skin lesions are very prominent, usually an erythematous rash or scaly plaques. A unique manifestation is necrolytic acral erythema (NAE), associated with zinc deficiency in patients with HCV infection[104], expressed on the dorsal aspects of the feet and extending to the toes. The treatment of NAE must combine oral zinc supplementation and HCV eradication. For more than 70 years, hypozincemia has also been associated with impaired night vision, initially in alcoholic cirrhosis and later on in various liver cirrhosis etiologies[103]. Importantly, one of the disturbing manifestations of zinc deficiency is an alteration in taste and smell. This may further exacerbate malnutrition due to reduced intake in the cirrhotic patient[37,105,106]. According to expert opinion, zinc supplementation can be administrated as a long-term treatment or at least until zinc blood level is within normal range with a recommended dose of 50 mg elemental zinc (220 mg zinc sulfate) once daily, in order to avoid copper malabsorption[37,103].

Magnesium is one of the most prevalent intracellular cations, secondary only to potassium. It has been demonstrated that chronic alcohol consumption impairs magnesium homeostasis affecting the brain, skeletal muscles, heart, and liver[107]. In addition, alcohol abuse can decrease nutritional intake and increase excretion of magnesium by an indirect effect on renal tubules[107]. Other factors that might lead to hypomagnesemia in cirrhotic patients are the poor absorption of magnesium in the distal jejunum, exacerbation of magnesium urinary excretion due to elevated aldosterone, growth hormone, and glucagon blood levels, and chronic administration of loop diuretics[108]. The association between magnesium serum levels and the presence of cirrhosis or its severity is questionable. Two studies have found that magnesium concentration was not significantly different between patients with and without liver cirrhosis and no significant correlation was found with the Child-Pugh score[109,110]. One study found magnesium levels were significantly lower in cirrhotic patients compared to controls, but there was no difference between compensated and decompensated patients[111]. Conversely, another study demonstrated a negative correlation between serum magnesium and Child-Pugh score, and lower levels of magnesium among patients with cirrhosis compared to controls[112]. This disagreement can result from the different disparity of etiologies for the liver disease. Nangliya *et al*[112] reported that the prevalence of alcoholic liver disease was over 40%, however, Agarwal *et al*[110] excluded chronic alcohol abuse. Another explanation is that magnesium is a cellular cation and perhaps its serum level does not reflect its true concentration. Koivisto *et al*[113] argued that serum magnesium concentrations

may be normal despite intracellular depletion and that a true estimation of magnesium depletion is through a magnesium loading test, since magnesium uptake is increased in the condition of magnesium depletion. In their study, 10 cirrhotic patients were compared to six healthy controls. No difference was found between pre-loading magnesium levels, but the uptake of magnesium (calculated as the delta between the IV administered dose and the amount excreted in 24-h urine) was more than four-fold higher among cirrhotics compared to controls[113]. Due to scarce data and conflicting evidence, there are no clear clinical practice recommendations regarding magnesium supplementation in cirrhotic patients.

While all patients with chronic liver disease are at risk for depletion of various fat-soluble, water-soluble vitamins and trace elements, the most well-recognized micronutrient deficiencies related to alcohol overuse are vitamin B12 (cyanocobalamin), vitamin A, vitamin D, thiamine (B1), folate (B9), pyridoxine (B6), and zinc[114,115]. Following supplementation, when needed, an annual check-up once stable levels are achieved may be recommended[116]. Administration of B-complex vitamins such as thiamine, folate, and pyridoxine is needed to prevent Wernicke encephalopathy[114], Korsakoff's syndrome, megaloblastic anaemia and neuropathies[116]. Moreover, and regardless of the etiology of cirrhosis, a consensus paper by the International Society for Hepatic Encephalopathy and Nitrogen Metabolism stated that since vitamin status is not easily assessed and since multivitamin supplementation is cheap and generally safe, use of short-term (2-wk course) oral vitamin supplements could be justified in patients with decompensated cirrhosis or at risk of malnutrition, and clinically apparent vitamin deficiencies should be treated specifically[117].

PROTEIN AND CALORIE INTAKE IN PATIENTS WITH OBESITY AND CIRRHOSIS

Decreased muscle mass and function are more prevalent among cirrhotic patients with either MHE or OHE compared to no HE, and protein malnutrition is an independent risk factor for both OHE (OR = 3.4; 95%CI: 1.4-6.9; *P* < 0.001) and MHE (OR = 2.15; 95%CI: 1.1-4.1; *P* = 0.002)[118]. In the late 90s, the recommendations for caloric intake were near-normal for patients with well-compensated liver cirrhosis [25-35 kcal/(kg•d)] and up to 30-40 kcal/(kg•d) in malnourished or critically ill cirrhotic patients[119]. The ESEPEN currently recommends a daily caloric intake of 35-40 kcal/(kg•d) for all cirrhotic patients regardless of their liver disease etiology or degree of liver function compensation[120], without a specific reference for cirrhotic patients with obesity, which may still be malnourished. However, weight loss should be encouraged in patients with obesity and well-compensated cirrhosis,

Table 2 Nutritional recommendations for cirrhotic patients with obesity

Nutrient	Recommendation
[1]Daily energy intake[117]	25-35 kcal/(kg•d) in patients with BMI 30-40 kg/m^2
	20-25 kcal/(kg•d) in patients with BMI > 40 kg/m^2
[2]Protein intake[119]	1.2-1.5 g/(kg•d)
[3]Micronutrients	Identify and correct micronutrient deficiencies
Fiber	25-45 g/d

[1]The recommended dietary pattern is for small, frequent meals evenly distributed throughout the day (every 3-6 h) with a late evening snack containing at least 50 g of complex carbohydrate. [2]Vegetable-protein based diets can also be beneficial in terms of caloric and fiber intake among overweight patients with cirrhosis who are attempting to lose weight. In addition, plant-based proteins are rich in branched-chain amino acids (BCAA)[117,127,128]. [3]Cirrhotic patients with obesity are at high risk for depletion of various fat-soluble, water-soluble vitamins and trace elements and should be supplemented appropriately. BMI: Body mass index.

nevertheless, over-restriction will result in endogenous muscle breakdown. Indeed, in later guidelines, Amodio *et al*[117] recommend careful monitoring alongside increased physical activity and caloric intake of 25-35 kcal/(kg•d) in patients with obesity (30-40 kg/m^2) and not less than 20-25 kcal/(kg•d) in patients with morbid obesity (> 40 kg/m^2). The recommended dietary pattern is for small, frequent meals evenly distributed throughout the day (every 3-6 h) with a late evening snack containing at least 50 g of complex carbohydrate[117].

The attitude toward daily protein intake has also changed. In the past, the daily protein intake of cirrhotic patients was tightly restricted to less than 0.8 g/(kg•d) due to concerns about the increased risk for HE. However, the tolerance of proteins in cirrhotic patients has been shown to be higher than previously believed[117,121], and the importance of substantial daily protein intake for sustaining adequate muscle mass is becoming more and more clear[117]. Córdoba *et al*[121] showed that administration of a low-protein diet [0.5 g/(kg•d)] worsened HE and exacerbated protein breakdown compared to a daily protein intake of 1.2 g/(kg•d).

Currently, the recommended daily protein intake is 1.2-1.5 g/(kg•d)[120]. For cirrhotic patients with obesity, a moderately hypocaloric diet must include an adequate amount of proteins [1.2-1.5 g/(kg•d)] in order to accomplish weight loss without muscle or lean mass depletion[117]. In cirrhotic patients, there is variable tolerance to different dietary proteins according to their source. Some uncontrolled studies have shown a better tolerance to vegetable proteins over meat proteins and to dairy proteins over mixed source proteins[122-124]. Interestingly, a 14-d casein-vegetable, high-protein, high-calorie diet was shown to improve mental performance and to decrease ammonia levels in 150 patients with overt HE[125]. The advantages of vegetable proteins may stem from the fact that they are rich in dietary fiber with prebiotic properties, which result in decreased transit time and intraluminal pH leading to increased fecal ammonia excretion. Moreover, vegetable proteins are rich in ornithine and arginine, that can facilitate clearance of ammonia through the urea cycle[126,127]. Vegetable-protein based diets can also be beneficial in terms of caloric and fiber intake among overweight patients with cirrhosis who are attempting to lose weight. In addition, plant-based proteins are rich in branched-chain amino acids (BCAA)[117,128,129]. In hepatic decompensation, there is a shift toward aromatic amino acids (AAA) (phenylalanine, tyrosine, and tryptophan) rather than BCAAs (isoleucine, leucine, and valine), however, AAA are assumed to cross the blood-brain barrier, act as false neurotransmitters, and induce HE[130,131]. BCAA supplementation can induce tolerability to meat protein and enable adequate protein intake[132]. Moreover, substituting meat with dairy or vegetable proteins along with BCAA supplements is better than reducing total proteins intake[117]. In a meta-analysis of 16 RCTs, BCAAs were found to have a beneficial effect on HE compared to placebo or best supportive care (diet, lactulose, or neomycin), however, no conclusions could be drawn regarding nutritional effects[133]. General nutritional recommendations in the cirrhotic patients with obesity are summarized in Table 2.

BARIATRIC SURGERY-INDUCED WEIGHT LOSS IN PATIENTS WITH OBESITY AND CIRRHOSIS: INDICATIONS AND CONTRAINDICATIONS

In recent years, parallel to the rapid and sharp increase in the prevalence of obesity[134], bariatric surgery has reached a rapid and deep penetration, as it is the only therapeutic means leading to long-term weight loss with consequent improvement of obesity-related comorbidities and patients' quality of life[135]. NAFLD has become an extremely frequent condition in recent years due to the obesity epidemic[136]. While NAFLD includes a spectrum of histologic features ranging from simple liver steatosis to steatohepatitis (NASH), liver fibrosis has been shown to be the main determinant of mortality linked to NASH in the long-term[137,138]. Obesity has been shown to be associated with an increased risk of primary liver cancer in several large epidemiological studies. Potential mechanisms responsible for this increased risk include the occurrence of NAFLD and type 2 diabetes[139]. Furthermore, there is evidence that the probability of clinical decompensation of liver cirrhosis is significantly

increased in the presence of obesity compared to overweight and lean subjects[140].

As a consequence, LT surgeons are faced more and more frequently with candidates for LT with morbid obesity[141]. Traditionally obesity has been considered a main risk factor for postoperative morbidity and mortality in the context of major and complex surgical procedures[142,143]. However, obesity is a heterogeneous disease including different conditions sharing a common denominator represented by an increased BMI. Indeed, there is clear epidemiological evidence that the presence of obesity has a protective effect against postoperative mortality and morbidity in the range of BMI below 35 kg/m². This phenomenon, known as the obesity paradox, has been attributed to the increased reserve of energy due to obesity that may confer an advantage in a condition of stress such as that after a major surgical procedure[144]. Furthermore, the presence of metabolic syndrome, which is associated with obesity but not obesity alone, is significantly associated with an increased risk of postoperative morbidity and mortality after major abdominal surgery. In spite of this, in many LT centers the presence of obesity, defined only on the basis of a BMI above 35 or 40 kg/m², is considered as a contraindication to LT[145]. Interestingly, the use of simple diagnostic tools such as anthropometric measures that include the psoas muscle diameter and visceral fat measure coupled with the presence of metabolic comorbidities may help to identify suitable candidates for LT among individuals with obesity without the need for massive weight loss[146,147].

Losing weight before LT remains an important goal but there is no consensus on how best to achieve it, especially in patients with obesity and cirrhosis. Many non-surgical methods can be used to allow patients to reach the desired BMI and be finally listed for LT, including increased physical activity, diet, and behavioral therapy as well as intragastric balloon[33]. Spengler *et al*[33] recently suggested the use of diet and lifestyle modifications in patients with compensated cirrhosis. For patients with compensated cirrhosis, bariatric surgery has also been proposed in preparation for LT[145-147]. Although this strategy may seem legitimate, bariatric surgery may lead to severe postoperative complications that a patient with a compromised liver function may not tolerate. Indeed, only patients with compensated cirrhosis are potential candidates for bariatric surgery as mortality in patients with decompensated cirrhosis would be inacceptable[148]. There is also evidence that bariatric surgery is associated with a significantly lower morbidity and mortality if an LT program is also present in the same hospital. This underlines the complexity of the patients that need multidisciplinary care from specialists in both hepatology and bariatrics.

The timing of surgery
The timing of bariatric surgery in LT candidates is imperative. While it has been suggested that bariatric surgery-induced weight loss may increase the candidacy for LT, only patients with compensated liver cirrhosis are potential candidates to bariatric surgery because the risk of mortality is too high in the setting of decompensated cirrhosis[148]. The primary indication for LT in patients with a conserved liver function is represented by HCC. While international guidelines for access to bariatric surgery include a disease-free interval of at least five years after the radical treatment of any malignancy, a different strategy to access a curative treatment such as LT for this specific category of patients seems to be legitimate. Only patients with the best chances of long-term survival should then be selected for this ambitious therapeutic strategy, which includes bariatric surgery to lose weight and control metabolic comorbidities followed by LT. Endovascular and/or percutaneous interventional radiology techniques should be concomitantly used to obtain temporary local control of HCC. Several preoperative scores such as the alpha-fetoprotein score[149], the Milan criteria, and Metroticket 2.0 may be useful tools to guide the selection of patients in order to obtain long-term survival rates after LT comparable to those obtained for patients with non-tumoral disease[150].

The option of performing bariatric surgery after LT offers several advantages including normal liver function with normal prothrombin time, the restoration of normal portal pressure, the possibility to optimize the nutritional status and the choice of procedure in relation to the presence or absence of metabolic syndrome. The presence of adhesions linked to transplantation is not a formal contraindication to the laparoscopic approach nor is the use of immunosuppressive drugs[151].

As obesity recurs often after LT, affecting the graft with NASH recurrence and patient survival with metabolic comorbidities, some authors evoked the seducing possibility of performing a bariatric procedure, namely sleeve gastrectomy (SG), at the same time as the LT[152]. However, a panel of argument still stands against this policy, these include: the potentially disastrous consequences of the most feared complication of SG, a leak at the top of the staple line in the setting of immunosuppressive treatment in a freshly transplanted patient; the reduced amount of calories that the transplanted patient would be able to assume with the diet spontaneously for several months after surgery; the impact of the two procedures on the body composition with the risk of excessive fatty free mass loss.

The choice of procedure
The presence of portal hypertension may render any bariatric procedure difficult and lead to life-threatening bleeding. The choice of the procedure should then take into account the risk of bleeding. A transjugular intrahepatic portosystemic shunt (TIPS) may be considered in patients with signs of portal hypertension such as platelets below 100000, a large spleen on CT scan, the presence of collateral circulation on the abdomen or on CT scan and esophageal varices. In case of doubt,

Table 3 Main factors involved in the choice of the bariatric procedure in the setting of liver transplantation

Factors to consider	SG	RYGB
Bleeding risk of bleeding[1]	Increased	Low
Endoscopic access to the biliary tree	Conserved	Impossible
Risk of portal vein thrombosis	Increased	Not affected
Risk of bariatric surgery induced liver failure	Absent	Low
Absorption of immunosuppressive drugs	Poorly affected	Decreased
Etiology of liver cirrhosis (NASH *vs* others)	Effective	Very Effective

[1]Consider measuring of portal pressure and use of transjugular intrahepatic portosystemic shunt in case of bariatric surgery before liver transplantation.
SG: Sleeve gastrectomy; RYGP: Roux-en-Y gastric bypass; NASH: Non-alcoholic steato hepatitis.

a direct measure of the portal pressure should be obtained. It should be considered that in the case of large spontaneous portosystemic shunts (larger than 1 cm), the TIPS might be inefficacious. The possibility to access the biliary tree endoscopically after LT should also be considered. Procedures with an intestinal bypass definitely limit the endoscopic access to the bile duct. Furthermore, some patients may need a biliodigestive reconstruction on a Roux-en-Y loop that should be then fashioned in addition to the intestinal bypass of the bariatric procedure. There is a risk of portal vein thrombosis in any abdominal procedure and bariatric surgery has been shown to increase this risk. The potential risk of liver complications due to the intestinal bypass is another point that needs attention. It is well recognized that bariatric procedures including an intestinal bypass may lead to liver failure. Although the mechanisms underlying this complication have not been completely elucidated, the abnormal intestinal microbiota derived from bacterial overgrowth may cause intestinal permeability defects that expose hosts to noxious gut-derived factors such as bacterial lipopolysaccharide, other toll-like receptor ligands, and toxic bile acids[153]. Interestingly, most, if not all, cases of liver failure reported in the literature include patients with a biliopancreatic diversion, in which the length of the bypassed intestine is very long, and the jejuno-ileal bypass, whereas only a few cases of liver failure in patients with Roux-en-Y gastric bypass (RYGP) have been reported[154]. The jejuno-ileal bypass was proscribed several decades ago and nowadays the Scopinaro's procedure is rarely performed. This is in line with the hypothesis that an altered microbiota may be a major determinant of increased intestinal permeability as the more distal is the bypass the more altered is the intestinal flora.

The absorption of immunosuppressive drugs should also be considered when choosing the most appropriate bariatric procedure. The absorption of drugs may not only be altered by the presence of an intestinal bypass but also by the changes in the intestinal pH linked to the suppression of hydrochloric acid secretion[155]. Finally, the etiology of liver disease (namely NASH *vs* viral infections and alcohol abuse), the presence of metabolic syndrome, and NASH recurrence on the graft in cases of patients that have already been transplanted are key factors to consider when choosing the type of bariatric procedure.

The three most common bariatric procedures currently performed worldwide are laparoscopic adjustable gastric banding (LAGB), the SG, and RYGP. Several factors are involved in the choice of the bariatric procedure in the setting of LT (Table 3). Although the LAGB has the advantage of being the easiest procedure with the lowest risk of bleeding, it also has the worse results in terms of weight loss with the highest rate of failure. Furthermore, the presence of foreign material around the mesogastric junction, especially in patients with esophageal varices, may cause problems. The SG is currently the most performed procedure[156,157] that offers several advantages, including the fact that no intestinal bypass is done, leaving the whole digestive tract accessible to endoscopic exploration, avoiding intestinal bypass related interference with the absorption of immunosuppressive drugs, and leaving the possibility to fashion a Roux-en-Y loop in the event of biliary complications after LT or in cases where a biliodigestive reconstruction is indicated such as in primary biliary cholangitis. However, in cases of portal hypertension, the division of the greater curvature vessels may expose to the risk of bleeding especially if portal pressure is increased and no TIPS has been put in place. Bleeding may be then difficult to control and should be feared as a life-threatening complication. The use of buttressing material for the division of the stomach may reduce significantly the risk of bleeding in these patients and should be used[158].

Of note is also the fact that the SG is followed by an extreme decrease in hydrochloric acid secretion, resulting in an altered pH, which may consequently interfere with immunosuppressive drug absorption[155]. Although short and medium-term results of SG on weight loss and control of metabolic syndrome indicate equivalent efficacy between the SG and the RYGP[159], long-term results of SG are still too scarce to drive definite conclusions on the equivalence of efficacy between the two procedures, especially in a NASH setting[160].

The RYGP has the advantage of a reduced risk of bleeding, as only the lesser curvature of the stomach, where bleeding may occur, needs to be dissected before gastric division. It is also more effective against metabolic complications of obesity that may be either present before or after LT[161]. Strong evidence in support of the preventive effect of RYGP against the occurrence of type

2 diabetes has come from several studies[162,163]. Recently Safwan *et al*[164] reported a series of 11 patients with a previous history of bariatric surgery that underwent LT and showed that biliary complications could be successfully addressed with a second Roux-en-Y loop in patients with an RYGP. One additional technical point concerns the use of the endoluminal tube to guide the shaping of the gastric pouch or sleeve. If the preoperative endoscopy has shown the presence of esophageal varices, in spite of appropriate endoscopic eradication, the orogastric tube may eventually injure the varices causing bleeding. For this reason, it should be better avoided as far as possible especially in patients with a low prothrombin time and platelet count. SG is also associated with an increased risk of portal thrombosis compared to RYGP in non-cirrhotic patients[165]. This may be a further concern, as liver cirrhosis is a risk factor for portal thrombosis in itself. The occurrence of portal thrombosis may further complicate the access to LT. The risk of dumping syndrome and hyperinsulinemic hypoglycemia may be further concerns of the RYGP.

In conclusion, with all of the potential that bariatric surgery has candidates to LT with morbid obesity, well-designed studies are still necessary to gain widespread acceptance from clinicians. Several points need to be clarified, including the most appropriate methods for accurate patient evaluation and selection, the optimal surgical procedure and its timing to maximize the efficiency of bariatric surgery in liver recipients with obesity. Only when this research is furthered to the point where the effectiveness of bariatric surgery is no longer in doubt, the latter will be included in the care of liver recipients with obesity for mainstream use. In the meantime, the policy of a case-by-case discussion involving a multidisciplinary team, including hepatologists and both LT and bariatric surgeons, seems to be justified.

CONCLUSION

With the recent epidemic of obesity, the coexistence of liver cirrhosis and obesity has become very frequent. The complex interplay between obesity and the liver especially in the setting of liver cirrhosis is a formidable challenge for current medicine that goes from the prevention of liver complications of obesity, screening for these complications in patients at risk to the management of patients with sarcopenia and end-stage liver disease. Bariatric surgery has shown promising results although evidence is still scarce.

REFERENCES

1   **Kleiner DE**, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ; Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**: 1313-1321 [PMID: 15915461 DOI: 10.1002/hep.20701]

2   **Welzel TM**, Graubard BI, Zeuzem S, El-Serag HB, Davila JA, McGlynn KA. Metabolic syndrome increases the risk of primary liver cancer in the United States: a study in the SEER-Medicare database. *Hepatology* 2011; **54**: 463-471 [PMID: 21538440 DOI: 10.1002/hep.24397]

3   **Starley BQ**, Calcagno CJ, Harrison SA. Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. *Hepatology* 2010; **51**: 1820-1832 [PMID: 20432259 DOI: 10.1002/hep.23594]

4   **Ascha MS**, Hanouneh IA, Lopez R, Tamimi TA, Feldstein AF, Zein NN. The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. *Hepatology* 2010; **51**: 1972-1978 [PMID: 20209604 DOI: 10.1002/hep.23527]

5   **Chalasani N**, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, Charlton M, Sanyal AJ. The diagnosis and management of non-alcoholic fatty liver disease: practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology* 2012; **55**: 2005-2023 [PMID: 22488764 DOI: 10.1002/hep.25762]

6   **Angulo P**. Long-term mortality in nonalcoholic fatty liver disease: is liver histology of any prognostic significance? *Hepatology* 2010; **51**: 373-375 [PMID: 20101746 DOI: 10.1002/hep.23521]

7   **Raynard B**, Balian A, Fallik D, Capron F, Bedossa P, Chaput JC, Naveau S. Risk factors of fibrosis in alcohol-induced liver disease. *Hepatology* 2002; **35**: 635-638 [PMID: 11870378 DOI: 10.1053/jhep.2002.31782]

8   **Ortiz V**, Berenguer M, Rayón JM, Carrasco D, Berenguer J. Contribution of obesity to hepatitis C-related fibrosis progression. *Am J Gastroenterol* 2002; **97**: 2408-2414 [PMID: 12358265 DOI: 10.1111/j.1572-0241.2002.05995.x]

9   **Bellentani S**, Saccoccio G, Masutti F, Crocè LS, Brandi G, Sasso F, Cristanini G, Tiribelli C. Prevalence of and risk factors for hepatic steatosis in Northern Italy. *Ann Intern Med* 2000; **132**: 112-117 [PMID: 10644271 DOI: 10.7326/0003-4819-132-2-200001180-00029]

10 **Ekstedt M**, Franzén LE, Holmqvist M, Bendtsen P, Mathiesen UL, Bodemar G, Kechagias S. Alcohol consumption is associated with progression of hepatic fibrosis in non-alcoholic fatty liver disease. *Scand J Gastroenterol* 2009; **44**: 366-374 [PMID: 19016382 DOI: 10.1080/00365520802555991]

11 **Liu B**, Balkwill A, Reeves G, Beral V; Million Women Study Collaborators. Body mass index and risk of liver cirrhosis in middle aged UK women: prospective study. *BMJ* 2010; **340**: c912 [PMID: 20223875 DOI: 10.1136/bmj.c912]

12 **Everhart JE**, Lok AS, Kim HY, Morgan TR, Lindsay KL, Chung RT, Bonkovsky HL, Ghany MG; HALT-C Trial Group. Weight-related effects on disease progression in the hepatitis C antiviral long-term treatment against cirrhosis trial. *Gastroenterology* 2009; **137**: 549-557 [PMID: 19445938 DOI: 10.1053/j.gastro.2009.05.007]

13 **Berzigotti A**, Abraldes JG. Impact of obesity and insulin-resistance on cirrhosis and portal hypertension. *Gastroenterol Hepatol* 2013; **36**: 527-533 [PMID: 23731977 DOI: 10.1016/j.gastrohep.2013.03.005]

14 **Berzigotti A**, Albillos A, Villanueva C, Genescá J, Ardevol A, Augustín S, Calleja JL, Bañares R, García-Pagán JC, Mesonero F, Bosch J; Ciberehd SportDiet Collaborative Group. Effects of an intensive lifestyle intervention program on portal hypertension in patients with cirrhosis and obesity: The SportDiet study. *Hepatology* 2017; **65**: 1293-1305 [PMID: 27997989 DOI: 10.1002/hep.28992]

15 **Ayala R**, Grande S, Bustelos R, Ribera C, García-Sesma A, Jimenez C, Moreno E, Martínez-López J. Obesity is an independent risk factor for pre-transplant portal vein thrombosis in liver recipients. *BMC Gastroenterol* 2012; **12**: 114 [PMID: 22909075 DOI: 10.1186/1471-230X-12-114]

16 **Allman-Farinelli MA**. Obesity and venous thrombosis: a review. *Semin Thromb Hemost* 2011; **37**: 903-907 [PMID: 22198855 DOI: 10.1055/s-0031-1297369]

17 **LaMattina JC**, Foley DP, Fernandez LA, Pirsch JD, Musat AI, D'Alessandro AM, Mezrich JD. Complications associated with liver transplantation in the obese recipient. *Clin Transplant* 2012; **26**: 910-918 [PMID: 22694047 DOI: 10.1111/j.1399-0012.2012.01669.x]

18 **Cruz-Jentoft AJ**, Baeyens JP, Bauer JM, Boirie Y, Cederholm T,

Landi F, Martin FC, Michel JP, Rolland Y, Schneider SM, Topinková E, Vandewoude M, Zamboni M; European Working Group on Sarcopenia in Older People. Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in Older People. *Age Ageing* 2010; **39**: 412-423 [PMID: 20392703 DOI: 10.1093/ageing/afq034]

19   **Rolland Y**, Lauwers-Cances V, Cristini C, Abellan van Kan G, Janssen I, Morley JE, Vellas B. Difficulties with physical function associated with obesity, sarcopenia, and sarcopenic-obesity in community-dwelling elderly women: the EPIDOS (EPIDemiologie de l'OSteoporose) Study. *Am J Clin Nutr* 2009; **89**: 1895-1900 [PMID: 19369381 DOI: 10.3945/ajcn.2008.26950]

20   **Koster A**, Ding J, Stenholm S, Caserotti P, Houston DK, Nicklas BJ, You T, Lee JS, Visser M, Newman AB, Schwartz AV, Cauley JA, Tylavsky FA, Goodpaster BH, Kritchevsky SB, Harris TB; Health ABC Study. Does the amount of fat mass predict age-related loss of lean mass, muscle strength, and muscle quality in older adults? *J Gerontol A Biol Sci Med Sci* 2011; **66**: 888-895 [PMID: 21572082 DOI: 10.1093/gerona/glr070]

21   **Beavers KM**, Hsu FC, Houston DK, Beavers DP, Harris TB, Hue TF, Kim LJ, Koster A, Penninx BW, Simonsick EM, Strotmeyer ES, Kritchevsky SB, Nicklas BJ; Health ABC Study. The role of metabolic syndrome, adiposity, and inflammation in physical performance in the Health ABC Study. *J Gerontol A Biol Sci Med Sci* 2013; **68**: 617-623 [PMID: 23109678 DOI: 10.1093/gerona/gls213]

22   **European Association for the Study of the Liver (EASL)**, European Association for the Study of Diabetes (EASD), European Association for the Study of Obesity (EASO). EASL-EASD-EASO Clinical Practice Guidelines for the Management of Non-Alcoholic Fatty Liver Disease. *Obes Facts* 2016; **9**: 65-90 [PMID: 27055256 DOI: 10.1159/000443344]

23   **Tovo CV**, Fernandes SA, Buss C, de Mattos AA. Sarcopenia and non-alcoholic fatty liver disease: Is there a relationship? A systematic review. *World J Hepatol* 2017; **9**: 326-332 [PMID: 28293382 DOI: 10.4254/wjh.v9.i6.326]

24   **Hong HC**, Hwang SY, Choi HY, Yoo HJ, Seo JA, Kim SG, Kim NH, Baik SH, Choi DS, Choi KM. Relationship between sarcopenia and nonalcoholic fatty liver disease: the Korean Sarcopenic Obesity Study. *Hepatology* 2014; **59**: 1772-1778 [PMID: 23996808 DOI: 10.1002/hep.26716]

25   **Lee YH**, Kim SU, Song K, Park JY, Kim DY, Ahn SH, Lee BW, Kang ES, Cha BS, Han KH. Sarcopenia is associated with significant liver fibrosis independently of obesity and insulin resistance in nonalcoholic fatty liver disease: Nationwide surveys (KNHANES 2008-2011). *Hepatology* 2016; **63**: 776-786 [PMID: 26638128 DOI: 10.1002/hep.28376]

26   **Shida T**, Akiyama K, Oh S, Sawai A, Isobe T, Okamoto Y, Ishige K, Mizokami Y, Yamagata K, Onizawa K, Tanaka H, Iijima H, Shoda J. Skeletal muscle mass to visceral fat area ratio is an important determinant affecting hepatic conditions of non-alcoholic fatty liver disease. *J Gastroenterol* 2018; **53**: 535-547 [PMID: 28791501 DOI: 10.1007/s00535-017-1377-3]

27   **Anand AC**. Nutrition and Muscle in Cirrhosis. *J Clin Exp Hepatol* 2017; **7**: 340-357 [PMID: 29234200 DOI: 10.1016/j.jceh.2017.11.001]

28   **Montano-Loza AJ**, Angulo P, Meza-Junco J, Prado CM, Sawyer MB, Beaumont C, Esfandiari N, Ma M, Baracos VE. Sarcopenic obesity and myosteatosis are associated with higher mortality in patients with cirrhosis. *J Cachexia Sarcopenia Muscle* 2016; **7**: 126-135 [PMID: 27493866 DOI: 10.1002/jcsm.12039]

29   **Hara N**, Iwasa M, Sugimoto R, Mifuji-Moroka R, Yoshikawa K, Terasaka E, Hattori A, Ishidome M, Kobayashi Y, Hasegawa H, Iwata K, Takei Y. Sarcopenia and Sarcopenic Obesity Are Prognostic Factors for Overall Survival in Patients with Cirrhosis. *Intern Med* 2016; **55**: 863-870 [PMID: 27086797 DOI: 10.2169/internalmedicine.55.5676]

30   **Carias S**, Castellanos AL, Vilchez V, Nair R, Dela Cruz AC, Watkins J, Barrett T, Trushar P, Esser K, Gedaly R. Nonalcoholic steatohepatitis is strongly associated with sarcopenic obesity in patients with cirrhosis undergoing liver transplant evaluation. *J Gastroenterol Hepatol* 2016; **31**: 628-633 [PMID: 26399838 DOI: 10.1111/jgh.13166]

31   **Kaido T**, Tamai Y, Hamaguchi Y, Okumura S, Kobayashi A, Shirai H, Yagi S, Kamo N, Hammad A, Inagaki N, Uemoto S. Effects of pretransplant sarcopenia and sequential changes in sarcopenic parameters after living donor liver transplantation. *Nutrition* 2017; **33**: 195-198 [PMID: 27649861 DOI: 10.1016/j.nut.2016.07.002]

32   **Hammad A**, Kaido T, Hamaguchi Y, Okumura S, Kobayashi A, Shirai H, Kamo N, Yagi S, Uemoto S. Impact of sarcopenic overweight on the outcomes after living donor liver transplantation. *Hepatobiliary Surg Nutr* 2017; **6**: 367-378 [PMID: 29312971 DOI: 10.21037/hbsn.2017.02.02]

33   **Spengler EK**, O'Leary JG, Te HS, Rogal S, Pillai AA, Al-Osaimi A, Desai A, Fleming JN, Ganger D, Seetharam A, Tsoulfas G, Montenovo M, Lai JC. Liver Transplantation in the Obese Cirrhotic Patient. *Transplantation* 2017; **101**: 2288-2296 [PMID: 28930104 DOI: 10.1097/TP.0000000000001794]

34   **Richards J**, Gunson B, Johnson J, Neuberger J. Weight gain and obesity after liver transplantation. *Transpl Int* 2005; **18**: 461-466 [PMID: 15773968 DOI: 10.1111/j.1432-2277.2004.00067.x]

35   **Schütz T**, Hudjetz H, Roske AE, Katzorke C, Kreymann G, Budde K, Fritsche L, Neumayer HH, Lochs H, Plauth M. Weight gain in long-term survivors of kidney or liver transplantation--another paradigm of sarcopenic obesity? *Nutrition* 2012; **28**: 378-383 [PMID: 22304858 DOI: 10.1016/j.nut.2011.07.019]

36   **Choudhary NS**, Saigal S, Saraf N, Mohanka R, Rastogi A, Goja S, Menon PB, Mishra S, Mittal A, Soin AS. Sarcopenic obesity with metabolic syndrome: a newly recognized entity following living donor liver transplantation. *Clin Transplant* 2015; **29**: 211-215 [PMID: 25594826 DOI: 10.1111/ctr.12505]

37   **Johnson TM**, Overgard EB, Cohen AE, DiBaise JK. Nutrition assessment and management in advanced liver disease. *Nutr Clin Pract* 2013; **28**: 15-29 [PMID: 23319353 DOI: 10.1177/0884533612469027]

38   **Floreani A**, Baragiotta A, Martines D, Naccarato R, D'odorico A. Plasma antioxidant levels in chronic cholestatic liver diseases. *Aliment Pharmacol Ther* 2000; **14**: 353-358 [PMID: 10735930 DOI: 10.1046/j.1365-2036.2000.00729.x]

39   **Hansen KE**, Johnson MG. An update on vitamin D for clinicians. *Curr Opin Endocrinol Diabetes Obes* 2016; **23**: 440-444 [PMID: 27653000 DOI: 10.1097/MED.0000000000000288]

40   **Stokes CS**, Volmer DA, Grünhage F, Lammert F. Vitamin D in chronic liver disease. *Liver Int* 2013; **33**: 338-352 [PMID: 23402606 DOI: 10.1111/liv.12106]

41   **Lim LY**, Chalasani N. Vitamin d deficiency in patients with chronic liver disease and cirrhosis. *Curr Gastroenterol Rep* 2012; **14**: 67-73 [PMID: 22113744 DOI: 10.1007/s11894-011-0231-7]

42   **Targher G**, Byrne CD. Lower 25-hydroxyvitamin D3 levels and increased risk of liver diseases: is there a causal link? *Endocrine* 2014; **47**: 3-4 [PMID: 24595898 DOI: 10.1007/s12020-014-0220-3]

43   **Stokes CS**, Krawczyk M, Reichel C, Lammert F, Grünhage F. Vitamin D deficiency is associated with mortality in patients with advanced liver cirrhosis. *Eur J Clin Invest* 2014; **44**: 176-183 [PMID: 24236541 DOI: 10.1111/eci.12205]

44   **Paternostro R**, Wagner D, Reiberger T, Mandorfer M, Schwarzer R, Ferlitsch M, Trauner M, Peck-Radosavljevic M, Ferlitsch A. Low 25-OH-vitamin D levels reflect hepatic dysfunction and are associated with mortality in patients with liver cirrhosis. *Wien Klin Wochenschr* 2017; **129**: 8-15 [PMID: 27888359 DOI: 10.1007/s00508-016-1127-1]

45   **Putz-Bankuti C**, Pilz S, Stojakovic T, Scharnagl H, Pieber TR, Trauner M, Obermayer-Pietsch B, Stauber RE. Association of 25-hydroxyvitamin D levels with liver dysfunction and mortality in chronic liver disease. *Liver Int* 2012; **32**: 845-851 [PMID: 22222013 DOI: 10.1111/j.1478-3231.2011.02735.x]

46   **Arteh J**, Narra S, Nair S. Prevalence of vitamin D deficiency in chronic liver disease. *Dig Dis Sci* 2010; **55**: 2624-2628 [PMID: 19960254 DOI: 10.1007/s10620-009-1069-9]

47   **Fisher L**, Fisher A. Vitamin D and parathyroid hormone in outpatients with noncholestatic chronic liver disease. *Clin Gastroenterol Hepatol*

2007; **5**: 513-520 [PMID: 17222588 DOI: 10.1016/j.cgh.2006.10.015]

48   **Jha AK**, Jha SK, Kumar A, Dayal VM, Jha SK. Effect of replenishment of vitamin D on survival in patients with decompensated liver cirrhosis: A prospective study. *World J Gastrointest Pathophysiol* 2017; **8**: 133-141 [PMID: 28868183 DOI: 10.4291/wjgp.v8.i3.133]

49   **Kitson MT**, Roberts SK. D-livering the message: the importance of vitamin D status in chronic liver disease. *J Hepatol* 2012; **57**: 897-909 [PMID: 22634121 DOI: 10.1016/j.jhep.2012.04.033]

50   **Reese PP**, Bloom RD, Feldman HI, Huverserian A, Thomasson A, Shults J, Hamano T, Goral S, Shaked A, Olthoff K, Rickels MR, Bleicher M, Leonard MB. Changes in vitamin D binding protein and vitamin D concentrations associated with liver transplantation. *Liver Int* 2012; **32**: 287-296 [PMID: 22098635 DOI: 10.1111/j.1478-3231.2011.02638.x]

51   **Skaaby T**, Husemoen LL, Borglykke A, Jørgensen T, Thuesen BH, Pisinger C, Schmidt LE, Linneberg A. Vitamin D status, liver enzymes, and incident liver disease and mortality: a general population study. *Endocrine* 2014; **47**: 213-220 [PMID: 24272594 DOI: 10.1007/s12020-013-0107-8]

52   **Wang JB**, Abnet CC, Chen W, Dawsey SM, Fan JH, Yin LY, Yin J, Major JM, Taylor PR, Qiao YL, Freedman ND. Association between serum 25(OH) vitamin D, incident liver cancer and chronic liver disease mortality in the Linxian Nutrition Intervention Trials: a nested case-control study. *Br J Cancer* 2013; **109**: 1997-2004 [PMID: 24008664 DOI: 10.1038/bjc.2013.546]

53   **Wortsman J**, Matsuoka LY, Chen TC, Lu Z, Holick MF. Decreased bioavailability of vitamin D in obesity. *Am J Clin Nutr* 2000; **72**: 690-693 [PMID: 10966885 DOI: 10.1093/ajcn/72.3.690]

54   **Stein EM**, Strain G, Sinha N, Ortiz D, Pomp A, Dakin G, McMahon DJ, Bockman R, Silverberg SJ. Vitamin D insufficiency prior to bariatric surgery: risk factors and a pilot treatment study. *Clin Endocrinol (Oxf)* 2009; **71**: 176-183 [PMID: 19018785 DOI: 10.1111/j.1365-2265.2008.03470.x]

55   **Chiu KC**, Chu A, Go VL, Saad MF. Hypovitaminosis D is associated with insulin resistance and beta cell dysfunction. *Am J Clin Nutr* 2004; **79**: 820-825 [PMID: 15113720 DOI: 10.1093/ajcn/79.5.820]

56   **Ganji V**, Zhang X, Shaikh N, Tangpricha V. Serum 25-hydroxyvitamin D concentrations are associated with prevalence of metabolic syndrome and various cardiometabolic risk factors in US children and adolescents based on assay-adjusted serum 25-hydroxyvitamin D data from NHANES 2001-2006. *Am J Clin Nutr* 2011; **94**: 225-233 [PMID: 21613551 DOI: 10.3945/ajcn.111.013516]

57   **Barchetta I**, De Bernardinis M, Capoccia D, Baroni MG, Fontana M, Fraioli A, Morini S, Leonetti F, Cavallo MG. Hypovitaminosis D is independently associated with metabolic syndrome in obese patients. *PLoS One* 2013; **8**: e68689 [PMID: 23935881 DOI: 10.1371/journal.pone.0068689]

58   **Shankar A**, Sabanayagam C, Kalidindi S. Serum 25-hydroxyvitamin d levels and prediabetes among subjects free of diabetes. *Diabetes Care* 2011; **34**: 1114-1119 [PMID: 21430085 DOI: 10.2337/dc10-1203]

59   **Knekt P**, Laaksonen M, Mattila C, Härkänen T, Marniemi J, Heliövaara M, Rissanen H, Montonen J, Reunanen A. Serum vitamin D and subsequent occurrence of type 2 diabetes. *Epidemiology* 2008; **19**: 666-671 [PMID: 18496468 DOI: 10.1097/EDE.0b013e318176b8ad]

60   **Kayaniyil S**, Retnakaran R, Harris SB, Vieth R, Knight JA, Gerstein HC, Perkins BA, Zinman B, Hanley AJ. Prospective associations of vitamin D with β-cell function and glycemia: the PROspective Metabolism and ISlet cell Evaluation (PROMISE) cohort study. *Diabetes* 2011; **60**: 2947-2953 [PMID: 21911752 DOI: 10.2337/db11-0465]

61   **Grimnes G**, Emaus N, Joakimsen RM, Figenschau Y, Jenssen T, Njølstad I, Schirmer H, Jorde R. Baseline serum 25-hydroxyvitamin D concentrations in the Tromsø Study 1994-95 and risk of developing type 2 diabetes mellitus during 11 years of follow-up. *Diabet Med* 2010; **27**: 1107-1115 [PMID: 20854377 DOI: 10.1111/j.1464-5491.2010.03092.x]

62   **Lamendola CA**, Ariel D, Feldman D, Reaven GM. Relations

between obesity, insulin resistance, and 25-hydroxyvitamin D. *Am J Clin Nutr* 2012; **95**: 1055-1059 [PMID: 22440850 DOI: 10.3945/ajcn.111.032060]

63   **Rajakumar K**, de las Heras J, Lee S, Holick MF, Arslanian SA. 25-hydroxyvitamin D concentrations and in vivo insulin sensitivity and β-cell function relative to insulin sensitivity in black and white youth. *Diabetes Care* 2012; **35**: 627-633 [PMID: 22238280 DOI: 10.2337/dc11-1825]

64   **Targher G**, Bertolini L, Scala L, Cigolini M, Zenari L, Falezza G, Arcaro G. Associations between serum 25-hydroxyvitamin D3 concentrations and liver histology in patients with non-alcoholic fatty liver disease. *Nutr Metab Cardiovasc Dis* 2007; **17**: 517-524 [PMID: 16928437 DOI: 10.1016/j.numecd.2006.04.002]

65   **Dasarathy J**, Periyalwar P, Allampati S, Bhinder V, Hawkins C, Brandt P, Khiyami A, McCullough AJ, Dasarathy S. Hypovitaminosis D is associated with increased whole body fat mass and greater severity of non-alcoholic fatty liver disease. *Liver Int* 2014; **34**: e118-e127 [PMID: 24118743 DOI: 10.1111/liv.12312]

66   **Jablonski KL**, Jovanovich A, Holmen J, Targher G, McFann K, Kendrick J, Chonchol M. Low 25-hydroxyvitamin D level is independently associated with non-alcoholic fatty liver disease. *Nutr Metab Cardiovasc Dis* 2013; **23**: 792-798 [PMID: 23415456 DOI: 10.1016/j.numecd.2012.12.006]

67   **Barchetta I**, Angelico F, Del Ben M, Baroni MG, Pozzilli P, Morini S, Cavallo MG. Strong association between non alcoholic fatty liver disease (NAFLD) and low 25(OH) vitamin D levels in an adult population with normal serum liver enzymes. *BMC Med* 2011; **9**: 85 [PMID: 21749681 DOI: 10.1186/1741-7015-9-85]

68   **Nelson JE**, Roth CL, Wilson LA, Yates KP, Aouizerat B, Morgan-Stevenson V, Whalen E, Hoofnagle A, Mason M, Gersuk V, Yeh MM, Kowdley KV. Vitamin D Deficiency Is Associated With Increased Risk of Non-alcoholic Steatohepatitis in Adults With Non-alcoholic Fatty Liver Disease: Possible Role for MAPK and NF-κB? *Am J Gastroenterol* 2016; **111**: 852-863 [PMID: 27002799 DOI: 10.1038/ajg.2016.51]

69   **Bril F**, Maximos M, Portillo-Sanchez P, Biernacki D, Lomonaco R, Subbarayan S, Correa M, Lo M, Suman A, Cusi K. Relationship of vitamin D with insulin resistance and disease severity in non-alcoholic steatohepatitis. *J Hepatol* 2015; **62**: 405-411 [PMID: 25195551 DOI: 10.1016/j.jhep.2014.08.040]

70   **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines: management of cholestatic liver diseases. *J Hepatol* 2009; **51**: 237-267 [PMID: 19501929 DOI: 10.1016/j.jhep.2009.04.009]

71   **Leslie WD**, Bernstein CN, Leboff MS; American Gastroenterological Association Clinical Practice Commitee. AGA technical review on osteoporosis in hepatic disorders. *Gastroenterology* 2003; **125**: 941-966 [PMID: 12949738 DOI: 10.1016/S0016-5085(03)01062-X]

72   **Barchetta I**, Cimini FA, Cavallo MG. Vitamin D Supplementation and Non-Alcoholic Fatty Liver Disease: Present and Future. *Nutrients* 2017; **9**: [PMID: 28906453 DOI: 10.3390/nu9091015]

73   **Kitson MT**, Pham A, Gordon A, Kemp W, Roberts SK. High-dose vitamin D supplementation and liver histology in NASH. *Gut* 2016; **65**: 717-718 [PMID: 26294696 DOI: 10.1136/gutjnl-2015-310417]

74   **Barchetta I**, Del Ben M, Angelico F, Di Martino M, Fraioli A, La Torre G, Saulle R, Perri L, Morini S, Tiberti C, Bertoccini L, Cimini FA, Panimolle F, Catalano C, Baroni MG, Cavallo MG. No effects of oral vitamin D supplementation on non-alcoholic fatty liver disease in patients with type 2 diabetes: a randomized, double-blind, placebo-controlled trial. *BMC Med* 2016; **14**: 92 [PMID: 27353492 DOI: 10.1186/s12916-016-0638-y]

75   **Nagpal J**, Pande JN, Bhartia A. A double-blind, randomized, placebo-controlled trial of the short-term effect of vitamin D3 supplementation on insulin sensitivity in apparently healthy, middle-aged, centrally obese men. *Diabet Med* 2009; **26**: 19-27 [PMID: 19125756 DOI: 10.1111/j.1464-5491.2008.02636.x]

76   **von Hurst PR**, Stonehouse W, Coad J. Vitamin D supplementation reduces insulin resistance in South Asian women living in New Zealand who are insulin resistant and vitamin D deficient - a randomised, placebo-controlled trial. *Br J Nutr* 2010; **103**: 549-555

[PMID: 19781131 DOI: 10.1017/S0007114509992017]

77   **Pittas AG**, Dawson-Hughes B, Li T, Van Dam RM, Willett WC, Manson JE, Hu FB. Vitamin D and calcium intake in relation to type 2 diabetes in women. *Diabetes Care* 2006; **29**: 650-656 [PMID: 16505521 DOI: 10.2337/diacare.29.03.06.dc05-1961]

78   **Stokes CS**, Grünhage F, Baus C, Volmer DA, Wagenpfeil S, Riemenschneider M, Lammert F. Vitamin D supplementation reduces depressive symptoms in patients with chronic liver disease. *Clin Nutr* 2016; **35**: 950-957 [PMID: 26212170 DOI: 10.1016/j.clnu.2015.07.004]

79   **Bitetto D**, Fabris C, Falleti E, Fornasiere E, Fumolo E, Fontanini E, Cussigh A, Occhino G, Baccarani U, Pirisi M, Toniutto P. Vitamin D and the risk of acute allograft rejection following human liver transplantation. *Liver Int* 2010; **30**: 417-444 [PMID: 19849776 DOI: 10.1111/j.1478-3231.2009.02154.x]

80   **Bjelakovic G**, Nikolova D, Bjelakovic M, Gluud C. Vitamin D supplementation for chronic liver diseases in adults. *Cochrane Database Syst Rev* 2017; **11**: CD011564 [PMID: 29099543 DOI: 10.1002/14651858.CD011564.pub2]

81   **Collier JD**, Ninkovic M, Compston JE. Guidelines on the management of osteoporosis associated with chronic liver disease. *Gut* 2002; **50 Suppl 1**: i1-i9 [PMID: 11788576 DOI: 10.1136/gut.50.suppl_1.i1]

82   **Dasarathy J**, Varghese R, Feldman A, Khiyami A, McCullough AJ, Dasarathy S. Patients with Nonalcoholic Fatty Liver Disease Have a Low Response Rate to Vitamin D Supplementation. *J Nutr* 2017; **147**: 1938-1946 [PMID: 28814531 DOI: 10.3945/jn.117.254292]

83   **Parola M**, Leonarduzzi G, Biasi F, Albano E, Biocca ME, Poli G, Dianzani MU. Vitamin E dietary supplementation protects against carbon tetrachloride-induced chronic liver damage and cirrhosis. *Hepatology* 1992; **16**: 1014-1021 [PMID: 1398481 DOI: 10.1002/hep.1840160426]

84   **Houglum K**, Venkataramani A, Lyche K, Chojkier M. A pilot study of the effects of d-alpha-tocopherol on hepatic stellate cell activation in chronic hepatitis C. *Gastroenterology* 1997; **113**: 1069-1073 [PMID: 9322499 DOI: 10.1053/gast.1997.v113.pm9322499]

85   **Sanyal AJ**, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, Neuschwander-Tetri BA, Lavine JE, Tonascia J, Unalp A, Van Natta M, Clark J, Brunt EM, Kleiner DE, Hoofnagle JH, Robuck PR; NASH CRN. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med* 2010; **362**: 1675-1685 [PMID: 20427778 DOI: 10.1056/NEJMoa0907929]

86   **Chalasani N**, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, Harrison SA, Brunt EM, Sanyal AJ. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. *Hepatology* 2018; **67**: 328-357 [PMID: 28714183 DOI: 10.1002/hep.29367]

87   **Muñoz SJ**, Heubi JE, Balistreri WF, Maddrey WC. Vitamin E deficiency in primary biliary cirrhosis: gastrointestinal malabsorption, frequency and relationship to other lipid-soluble vitamins. *Hepatology* 1989; **9**: 525-531 [PMID: 2925155 DOI: 10.1002/hep.1840090403]

88   **Jeffrey GP**, Muller DP, Burroughs AK, Matthews S, Kemp C, Epstein O, Metcalfe TA, Southam E, Tazir-Melboucy M, Thomas PK. Vitamin E deficiency and its clinical significance in adults with primary biliary cirrhosis and other forms of chronic liver disease. *J Hepatol* 1987; **4**: 307-317 [PMID: 3036938 DOI: 10.1016/S0168-8278(87)80539-1]

89   **Leo MA**, Rosman AS, Lieber CS. Differential depletion of carotenoids and tocopherol in liver disease. *Hepatology* 1993; **17**: 977-986 [PMID: 8514270 DOI: 10.1002/hep.1840170606]

90   **Grüngreiff K**, Reinhold D, Wedemeyer H. The role of zinc in liver cirrhosis. *Ann Hepatol* 2016; **15**: 7-16 [PMID: 26626635 DOI: 10.5604/16652681.1184191]

91   **Ma J**, Betts NM. Zinc and copper intakes and their major food sources for older adults in the 1994-96 continuing survey of food intakes by individuals (CSFII). *J Nutr* 2000; **130**: 2838-2843 [PMID: 11053529 DOI: 10.1093/jn/130.11.2838]

92   **Huskisson E**, Maggini S, Ruf M. The role of vitamins and minerals in energy metabolism and well-being. *J Int Med Res* 2007; **35**: 277-289 [PMID: 17593855 DOI: 10.1177/147323000703500301]

93   **Gibson RS**, King JC, Lowe N. A Review of Dietary Zinc Recommendations. *Food Nutr Bull* 2016; **37**: 443-460 [PMID: 27312357 DOI: 10.1177/0379572116652252]

94   **Himoto T**, Masaki T. Associations between Zinc Deficiency and Metabolic Abnormalities in Patients with Chronic Liver Disease. *Nutrients* 2018; **10**: [PMID: 29342898 DOI: 10.3390/nu10010088]

95   **Chiba M**, Katayama K, Takeda R, Morita R, Iwahashi K, Onishi Y, Kita H, Nishio A, Kanno T, Saito T, Maeda K, Naito M, Michida T, Ito T. Diuretics aggravate zinc deficiency in patients with liver cirrhosis by increasing zinc excretion in urine. *Hepatol Res* 2013; **43**: 365-373 [PMID: 22994500 DOI: 10.1111/j.1872-034X.2012.01093.x]

96   **Nusrat S**, Khan MS, Fazili J, Madhoun MF. Cirrhosis and its complications: evidence based treatment. *World J Gastroenterol* 2014; **20**: 5442-5460 [PMID: 24833875 DOI: 10.3748/wjg.v20.i18.5442]

97   **Van der Rijt CC**, Schalm SW, Schat H, Foeken K, De Jong G. Overt hepatic encephalopathy precipitated by zinc deficiency. *Gastroenterology* 1991; **100**: 1114-1118 [PMID: 2001810 DOI: 10.1016/0016-5085(91)90290-2]

98   **Rabbani P**, Prasad AS. Plasma ammonia and liver ornithine transcarbamoylase activity in zinc-deficient rats. *Am J Physiol* 1978; **235**: E203-E206 [PMID: 686166 DOI: 10.1152/ajpendo.1978.235.2.E203]

99   **Katayama K**, Saito M, Kawaguchi T, Endo R, Sawara K, Nishiguchi S, Kato A, Kohgo H, Suzuki K, Sakaida I, Ueno Y, Habu D, Ito T, Moriwaki H, Suzuki K. Effect of zinc on liver cirrhosis with hyperammonemia: a preliminary randomized, placebo-controlled double-blind trial. *Nutrition* 2014; **30**: 1409-1414 [PMID: 25280421 DOI: 10.1016/j.nut.2014.04.018]

100   **Marchesini G**, Fabbri A, Bianchi G, Brizi M, Zoli M. Zinc supplementation and amino acid-nitrogen metabolism in patients with advanced cirrhosis. *Hepatology* 1996; **23**: 1084-1092 [PMID: 8621138 DOI: 10.1053/jhep.1996.v23.pm0008621138]

101   **Takuma Y**, Nouso K, Makino Y, Hayashi M, Takahashi H. Clinical trial: oral zinc in hepatic encephalopathy. *Aliment Pharmacol Ther* 2010; **32**: 1080-1090 [PMID: 20822500 DOI: 10.1111/j.1365-2036.2010.04448.x]

102   **Mousa N**, Abdel-Razik A, Zaher A, Hamed M, Shiha G, Effat N, Elbaz S, Elhelaly R, Hafez M, El-Wakeel N, Eldars W. The role of antioxidants and zinc in minimal hepatic encephalopathy: a randomized trial. *Therap Adv Gastroenterol* 2016; **9**: 684-691 [PMID: 27582881 DOI: 10.1177/1756283X16645049]

103   **Mohammad MK**, Zhou Z, Cave M, Barve A, McClain CJ. Zinc and liver disease. *Nutr Clin Pract* 2012; **27**: 8-20 [PMID: 22307488 DOI: 10.1177/0884533611433534]

104   **Tabibian JH**, Gerstenblith MR, Tedford RJ, Junkins-Hopkins JM, Abuav R. Necrolytic acral erythema as a cutaneous marker of hepatitis C: report of two cases and review. *Dig Dis Sci* 2010; **55**: 2735-2743 [PMID: 20499177 DOI: 10.1007/s10620-010-1273-7]

105   **Pimentel CF**, Lai M. Nutrition Interventions for Chronic Liver Diseases and Nonalcoholic Fatty Liver Disease. *Med Clin North Am* 2016; **100**: 1303-1327 [PMID: 27745596 DOI: 10.1016/j.mcna.2016.06.010]

106   **Tandon P**, Raman M, Mourtzakis M, Merli M. A practical approach to nutritional screening and assessment in cirrhosis. *Hepatology* 2017; **65**: 1044-1057 [PMID: 28027577 DOI: 10.1002/hep.29003]

107   **Romani AM**. Magnesium homeostasis and alcohol consumption. *Magnes Res* 2008; **21**: 197-204 [PMID: 19271417]

108   **Cohen L**. Magnesium and liver cirrhosis: a hypothesis. *Magnesium* 1985; **4**: 1-4 [PMID: 4033201]

109   **Rahelić D**, Kujundzić M, Romić Z, Brkić K, Petrovecki M. Serum concentration of zinc, copper, manganese and magnesium in patients with liver cirrhosis. *Coll Antropol* 2006; **30**: 523-528 [PMID: 17058518]

110   **Agarwal A**, Avarebeel S, Choudhary NS, Goudar M, Tejaswini CJ. Correlation of Trace Elements in Patients of Chronic Liver Disease with Respect to Child- Turcotte- Pugh Scoring System. *J Clin Diagn Res* 2017; **11**: OC25-OC28 [PMID: 29207755 DOI: 10.7860/

JCDR/2017/26519.10655]

111 **Kar K**, Dasgupta A, Vijaya Bhaskar M, Sudhakar K. Alteration of micronutrient status in compensated and decompensated liver cirrhosis. *Indian J Clin Biochem* 2014; **29**: 232-237 [PMID: 24757308 DOI: 10.1007/s12291-013-0349-5]

112 **Nangliya V**, Sharma A, Yadav D, Sunder S, Nijhawan S, Mishra S. Study of trace elements in liver cirrhosis patients and their role in prognosis of disease. *Biol Trace Elem Res* 2015; **165**: 35-40 [PMID: 25613584 DOI: 10.1007/s12011-015-0237-3]

113 **Koivisto M**, Valta P, Höckerstedt K, Lindgren L. Magnesium depletion in chronic terminal liver cirrhosis. *Clin Transplant* 2002; **16**: 325-328 [PMID: 12225427 DOI: 10.1034/j.1399-0012.2002.01141.x]

114 **Chao A**, Waitzberg D, de Jesus RP, Bueno AA, Kha V, Allen K, Kappus M, Medici V. Malnutrition and Nutritional Support in Alcoholic Liver Disease: a Review. *Curr Gastroenterol Rep* 2016; **18**: 65 [PMID: 27787787 DOI: 10.1007/s11894-016-0539-4]

115 **O'Shea RS**, Dasarathy S, McCullough AJ; Practice Guideline Committee of the American Association for the Study of Liver Diseases; Practice Parameters Committee of the American College of Gastroenterology. Alcoholic liver disease. *Hepatology* 2010; **51**: 307-328 [PMID: 20034030 DOI: 10.1002/hep.23258]

116 **Rossi RE**, Conte D, Massironi S. Diagnosis and treatment of nutritional deficiencies in alcoholic liver disease: Overview of available evidence and open issues. *Dig Liver Dis* 2015; **47**: 819-825 [PMID: 26164399 DOI: 10.1016/j.dld.2015.05.021]

117 **Amodio P**, Bemeur C, Butterworth R, Cordoba J, Kato A, Montagnese S, Uribe M, Vilstrup H, Morgan MY. The nutritional management of hepatic encephalopathy in patients with cirrhosis: International Society for Hepatic Encephalopathy and Nitrogen Metabolism Consensus. *Hepatology* 2013; **58**: 325-336 [PMID: 23471642 DOI: 10.1002/hep.26370]

118 **Merli M**, Giusto M, Lucidi C, Giannelli V, Pentassuglio I, Di Gregorio V, Lattanzi B, Riggio O. Muscle depletion increases the risk of overt and minimal hepatic encephalopathy: results of a prospective study. *Metab Brain Dis* 2013; 28: 281-284 [PMID: 23224378 DOI: 10.1007/s11011-012-9365-z]

119 **Plauth M**, Merli M, Kondrup J, Weimann A, Ferenci P, Müller MJ; ESPEN Consensus Group. ESPEN guidelines for nutrition in liver disease and transplantation. *Clin Nutr* 1997; **16**: 43-55 [PMID: 16844569 DOI: 10.1016/S0261-5614(97)80022-2]

120 **Plauth M**, Cabré E, Riggio O, Assis-Camilo M, Pirlich M, Kondrup J; DGEM (German Society for Nutritional Medicine), Ferenci P, Holm E, Vom Dahl S, Müller MJ, Nolte W; ESPEN (European Society for Parenteral and Enteral Nutrition). ESPEN Guidelines on Enteral Nutrition: Liver disease. *Clin Nutr* 2006; **25**: 285-294 [PMID: 16707194 DOI: 10.1016/j.clnu.2006.01.018]

121 **Córdoba J**, López-Hellín J, Planas M, Sabín P, Sanpedro F, Castro F, Esteban R, Guardia J. Normal protein diet for episodic hepatic encephalopathy: results of a randomized study. *J Hepatol* 2004; **41**: 38-43 [PMID: 15246205 DOI: 10.1016/j.jhep.2004.03.023]

122 **BESSMAN AN**, MIRICK GS. Blood ammonia levels following the ingestion of casein and whole blood. *J Clin Invest* 1958; **37**: 990-998 [PMID: 13563627 DOI: 10.1172/JCI103695]

123 **Fenton JC**, Knight EJ, Humpherson PL. Milk-and-cheese diet in portal-systemic encephalopathy. *Lancet* 1966; **1**: 164-166 [PMID: 4159095 DOI: 10.1016/S0140-6736(66)90696-9]

124 **Greenberger NJ**, Carley J, Schenker S, Bettinger I, Stamnes C, Beyer P. Effect of vegetable and animal protein diets in chronic hepatic encephalopathy. *Am J Dig Dis* 1977; **22**: 845-855 [PMID: 335882 DOI: 10.1007/BF01076158]

125 **Gheorghe L**, Iacob R, Vădan R, Iacob S, Gheorghe C. Improvement of hepatic encephalopathy using a modified high-calorie high-protein diet. *Rom J Gastroenterol* 2005; **14**: 231-238 [PMID: 16200232]

126 **Uribe M**, Dibildox M, Malpica S, Guillermo E, Villallobos A, Nieto L, Vargas F, Garcia Ramos G. Beneficial effect of vegetable protein diet supplemented with psyllium plantago in patients with hepatic encephalopathy and diabetes mellitus. *Gastroenterology* 1985; **88**: 901-907 [PMID: 2982694 DOI: 10.1016/S0016-5085(85)80006-8]

127 **Weber FL Jr**, Minco D, Fresard KM, Banwell JG. Effects of vegetable diets on nitrogen metabolism in cirrhotic subjects.

Gastroenterology 1985; **89**: 538-544 [PMID: 2991068 DOI: 10.1016/0016-5085(85)90448-2]

128 **Uribe M**, Márquez MA, Garcia Ramos G, Ramos-Uribe MH, Vargas F, Villalobos A, Ramos C. Treatment of chronic portal-systemic encephalopathy with vegetable and animal protein diets. A controlled crossover study. *Dig Dis Sci* 1982; **27**: 1109-1116 [PMID: 6756833 DOI: 10.1007/BF01391449]

129 **Shekib LA**, Zoueil ME, Youssef MM, Mohamed SW. Amino acid composition and In Vitro digestibility of lentil and rice proteins and their mixture (Koshary). *Food Chem* 1986; **20**: 61-67 [DOI: 10.1016/0308-8146(86)90167-6]

130 **Silk DB**. Branched chain amino acids in liver disease: fact or fantasy? *Gut* 1986; **27** Suppl 1: 103-110 [PMID: 3098636 DOI: 10.1136/gut.27.Suppl_1.103]

131 **Fischer JE**, Baldessarini RJ. False neurotransmitters and hepatic failure. *Lancet* 1971; **2**: 75-80 [PMID: 4103986 DOI: 10.1016/S0140-6736(71)92048-4]

132 **Dam G**, Ott P, Aagaard NK, Vilstrup H. Branched-chain amino acids and muscle ammonia detoxification in cirrhosis. *Metab Brain Dis* 2013; **28**: 217-220 [PMID: 23315357 DOI: 10.1007/s11011-013-9377-3]

133 **Gluud LL**, Dam G, Les I, Marchesini G, Borre M, Aagaard NK, Vilstrup H. Branched-chain amino acids for people with hepatic encephalopathy. *Cochrane Database Syst Rev* 2017; **5**: CD001939 [PMID: 28518283 DOI: 10.1002/14651858.CD001939.pub4]

134 **NCD Risk Factor Collaboration (NCD-RisC)**. Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19·2 million participants. *Lancet* 2016; **387**: 1377-1396 [PMID: 27115820 DOI: 10.1016/S0140-6736(16)30054-X]

135 **Angrisani L**, Santonicola A, Iovino P, Formisano G, Buchwald H, Scopinaro N. Bariatric Surgery Worldwide 2013. *Obes Surg* 2015; **25**: 1822-1832 [PMID: 25835983 DOI: 10.1007/s11695-015-1657-z]

136 **Diehl AM**, Day C. Cause, Pathogenesis, and Treatment of Nonalcoholic Steatohepatitis. *N Engl J Med* 2017; **377**: 2063-2072 [PMID: 29166236 DOI: 10.1056/NEJMra1503519]

137 **Angulo P**, Kleiner DE, Dam-Larsen S, Adams LA, Bjornsson ES, Charatcharoenwitthaya P, Mills PR, Keach JC, Lafferty HD, Stahler A, Haflidadottir S, Bendtsen F. Liver Fibrosis, but No Other Histologic Features, Is Associated With Long-term Outcomes of Patients With Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2015; **149**: 389-97.e10 [PMID: 25935633 DOI: 10.1053/j.gastro.2015.04.043]

138 **Ekstedt M**, Hagström H, Nasr P, Fredrikson M, Stål P, Kechagias S, Hultcrantz R. Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up. *Hepatology* 2015; **61**: 1547-1554 [PMID: 25125077 DOI: 10.1002/hep.27368]

139 **Bhaskaran K**, Douglas I, Forbes H, dos-Santos-Silva I, Leon DA, Smeeth L. Body-mass index and risk of 22 specific cancers: a population-based cohort study of 5·24 million UK adults. *Lancet* 2014; **384**: 755-765 [PMID: 25129328 DOI: 10.1016/S0140-6736(14)60892-8]

140 **Watt KD**. Reducing the load: the evolution and management of obesity and nonalcoholic steatohepatitis before liver transplantation. *Liver Transpl* 2012; **18** Suppl 2: S52-S58 [PMID: 22821716 DOI: 10.1002/lt.23515]

141 **Iannelli A**. Bariatric surgery and liver transplant. *Liver Transpl* 2017; **23**: 1369-1370 [PMID: 28945954 DOI: 10.1002/lt.24948]

142 **Saab S**, Lalezari D, Pruthi P, Alper T, Tong MJ. The impact of obesity on patient survival in liver transplant recipients: a meta-analysis. *Liver Int* 2015; **35**: 164-170 [PMID: 24313970 DOI: 10.1111/liv.12431]

143 **Perez-Protto SE**, Quintini C, Reynolds LF, You J, Cywinski JB, Sessler DI, Miller C. Comparable graft and patient survival in lean and obese liver transplant recipients. *Liver Transpl* 2013; **19**: 907-915 [PMID: 23744721 DOI: 10.1002/lt.23680]

144 **Karagozian R**, Bhardwaj G, Wakefield DB, Baffy G. Obesity paradox in advanced liver disease: obesity is associated with lower mortality in hospitalized patients with cirrhosis. *Liver Int* 2016; **36**: 1450-1456 [PMID: 27037497 DOI: 10.1111/liv.13137]

145 **Terjimanian MN**, Harbaugh CM, Hussain A, Olugbade KO Jr, Waits SA, Wang SC, Sonnenday CJ, Englesbe MJ. Abdominal adiposity, body composition and survival after liver transplantation. *Clin Transplant* 2016; **30**: 289-294 [PMID: 26717257 DOI: 10.1111/ctr.12688]

146 **Hamaguchi Y**, Kaido T, Okumura S, Kobayashi A, Shirai H, Yagi S, Kamo N, Okajima H, Uemoto S. Impact of Skeletal Muscle Mass Index, Intramuscular Adipose Tissue Content, and Visceral to Subcutaneous Adipose Tissue Area Ratio on Early Mortality of Living Donor Liver Transplantation. *Transplantation* 2017; **101**: 565-574 [PMID: 27926595 DOI: 10.1097/TP.0000000000001587]

147 **Lazzati A**, Iannelli A, Schneck AS, Nelson AC, Katsahian S, Gugenheim J, Azoulay D. Bariatric surgery and liver transplantation: a systematic review a new frontier for bariatric surgery. *Obes Surg* 2015; **25**: 134-142 [PMID: 25337867 DOI: 10.1007/s11695-014-1430-8]

148 **Mosko JD**, Nguyen GC. Increased perioperative mortality following bariatric surgery among patients with cirrhosis. *Clin Gastroenterol Hepatol* 2011; **9**: 897-901 [PMID: 21782772 DOI: 10.1016/j.cgh.2011.07.007]

149 **Duvoux C**, Roudot-Thoraval F, Decaens T, Pessione F, Badran H, Piardi T, Francoz C, Compagnon P, Vanlemmens C, Dumortier J, Dharancy S, Gugenheim J, Bernard PH, Adam R, Radenne S, Muscari F, Conti F, Hardwigsen J, Pageaux GP, Chazouillères O, Salame E, Hilleret MN, Lebray P, Abergel A, Debette-Gratien M, Kluger MD, Mallat A, Azoulay D, Cherqui D; Liver Transplantation French Study Group. Liver transplantation for hepatocellular carcinoma: a model including α-fetoprotein improves the performance of Milan criteria. *Gastroenterology* 2012; **143**: 986-94.e3; quiz e14-145 [PMID: 22750200 DOI: 10.1053/j.gastro.2012.05.052]

150 **Mazzaferro V**, Sposito C, Zhou J, Pinna AD, De Carlis L, Fan J, Cescon M, Di Sandro S, Yi-Feng H, Lauterio A, Bongini M, Cucchetti A. Metroticket 2.0 Model for Analysis of Competing Risks of Death After Liver Transplantation for Hepatocellular Carcinoma. *Gastroenterology* 2018; **154**: 128-139 [PMID: 28989060 DOI: 10.1053/j.gastro.2017.09.025]

151 **Al-Nowaylati AR**, Al-Haddad BJ, Dorman RB, Alsaied OA, Lake JR, Chinnakotla S, Slusarek BM, Sampson BK, Ikramuddin S, Buchwald H, Leslie DB. Gastric bypass after liver transplantation. *Liver Transpl* 2013; **19**: 1324-1329 [PMID: 24039124 DOI: 10.1002/lt.23734]

152 **Heimbach JK**, Watt KD, Poterucha JJ, Ziller NF, Cecco SD, Charlton MR, Hay JE, Wiesner RH, Sanchez W, Rosen CB, Swain JM. Combined liver transplantation and gastric sleeve resection for patients with medically complicated obesity and end-stage liver disease. *Am J Transplant* 2013; **13**: 363-368 [PMID: 23137119 DOI: 10.1111/j.1600-6143.2012.04318.x]

153 **Prakash G**, Drenick EJ, Wexler H, DeLucia L, Finegold SM. Microbial flora in the bypassed jejunum of patients with bilopancreatic bypass for obesity. *Am J Clin Nutr* 1987; **46**: 273-276 [PMID: 3618530 DOI: 10.1093/ajcn/46.2.273]

154 **D'Albuquerque LA**, Gonzalez AM, Wahle RC, de Oliveira Souza E, Mancero JM, de Oliveira e Silva A. Liver transplantation for subacute hepatocellular failure due to massive steatohepatitis after bariatric surgery. *Liver Transpl* 2008; **14**: 881-885 [PMID: 18508357 DOI: 10.1002/lt.21472]

155 **Rogers CC**, Alloway RR, Alexander JW, Cardi M, Trofe J, Vinks AA. Pharmacokinetics of mycophenolic acid, tacrolimus and sirolimus after gastric bypass surgery in end-stage renal disease and transplant patients: a pilot study. *Clin Transplant* 2008; **22**: 281-291 [PMID: 18482049 DOI: 10.1111/j.1399-0012.2007.00783.x]

156 **Clapp B**, Wynn M, Martyn C, Foster C, O'Dell M, Tyroch A. Long term (7 or more years) outcomes of the sleeve gastrectomy: a meta-analysis. *Surg Obes Relat Dis* 2018; **14**: 741-747 [PMID: 29625744 DOI: 10.1016/j.soard.2018.02.027]

157 **Noel P**, Nedelcu M, Eddbali I, Manos T, Gagner M. What are the long-term results 8 years after sleeve gastrectomy? *Surg Obes Relat Dis* 2017; **13**: 1110-1115 [PMID: 28755888 DOI: 10.1016/j.soard.2017.03.007]

158 **Berger ER**, Clements RH, Morton JM, Huffman KM, Wolfe BM, Nguyen NT, Ko CY, Hutter MM. The Impact of Different Surgical Techniques on Outcomes in Laparoscopic Sleeve Gastrectomies: The First Report from the Metabolic and Bariatric Surgery Accreditation and Quality Improvement Program (MBSAQIP). *Ann Surg* 2016; **264**: 464-473 [PMID: 27433904 DOI: 10.1097/SLA.0000000000001851]

159 **Peterli R**, Wölnerhanssen BK, Vetter D, Nett P, Gass M, Borbély Y, Peters T, Schiesser M, Schultes B, Beglinger C, Drewe J, Bueter M. Laparoscopic Sleeve Gastrectomy Versus Roux-Y-Gastric Bypass for Morbid Obesity-3-Year Outcomes of the Prospective Randomized Swiss Multicenter Bypass Or Sleeve Study (SM-BOSS). *Ann Surg* 2017; **265**: 466-473 [PMID: 28170356 DOI: 10.1097/SLA.0000000000001929]

160 **Schneck AS**, Anty R, Patouraux S, Bonnafous S, Rousseau D, Lebeaupin C, Bailly-Maitre B, Sans A, Tran A, Gugenheim J, Iannelli A, Gual P. Roux-En Y Gastric Bypass Results in Long-Term Remission of Hepatocyte Apoptosis and Hepatic Histological Features of Non-alcoholic Steatohepatitis. *Front Physiol* 2016; **7**: 344 [PMID: 27594839 DOI: 10.3389/fphys.2016.00344]

161 **Geerts A**, Darius T, Chapelle T, Roeyen G, Francque S, Libbrecht L, Nevens F, Pirenne J, Troisi R. The multicenter Belgian survey on liver transplantation for hepatocellular failure after bariatric surgery. *Transplant Proc* 2010; **42**: 4395-4398 [PMID: 21168706 DOI: 10.1016/j.transproceed.2010.07.010]

162 **Sjöström L**. Review of the key results from the Swedish Obese Subjects (SOS) trial - a prospective controlled intervention study of bariatric surgery. *J Intern Med* 2013; **273**: 219-234 [PMID: 23163728 DOI: 10.1111/joim.12012]

163 **Adams TD**, Davidson LE, Litwin SE, Kim J, Kolotkin RL, Nanjee MN, Gutierrez JM, Frogley SJ, Ibele AR, Brinton EA, Hopkins PN, McKinlay R, Simper SC, Hunt SC. Weight and Metabolic Outcomes 12 Years after Gastric Bypass. *N Engl J Med* 2017; **377**: 1143-1155 [PMID: 28930514 DOI: 10.1056/NEJMoa1700459]

164 **Safwan M**, Collins KM, Aboujjoud MS, Salgia R. Outcome of liver transplantation in patients with prior bariatric surgery. *Liver Transpl* 2017; **23**: 1415-1421 [PMID: 28752920 DOI: 10.1002/lt.24832]

165 **Shaheen O**, Siejka J, Thatigotla B, Pham DT. A systematic review of portomesenteric vein thrombosis after sleeve gastrectomy. *Surg Obes Relat Dis* 2017; **13**: 1422-1431 [PMID: 28526434 DOI: 10.1016/j.soard.2017.03.015]

Host genetic factors affecting hepatitis B infection outcomes: Insights from genome-wide association studies

Izzet Mehmet Akcay, Seyma Katrinli, Kamil Ozdil, Gizem Dinler Doganay, Levent Doganay

Izzet Mehmet Akcay, Seyma Katrinli, Gizem Dinler Doganay, Department of Molecular Biology and Genetics, Istanbul Technical University, Istanbul 34469, Turkey

Kamil Ozdil, Levent Doganay, Department of Gastroenterology and Hepatology, Umraniye Teaching and Research Hospital, Istanbul 34764, Turkey

ORCID number: Izzet Mehmet Akcay (0000-0001-6364-0062); Seyma Katrinli (0000-0003-1575-8642); Kamil Ozdil (0000-0003-2556-3064); Gizem Dinler Doganay (0000-0002-3586-1287); Levent Doganay (0000-0002-2263-6689).

Correspondence to: Levent Doganay, MD, Associate Professor, Doctor, Department of Gastroenterology and Hepatology, Umraniye Teaching and Research Hospital, Umraniye, Istanbul 34764, Turkey. levent.doganay@saglik.gov.tr
Telephone: +90-216-6321818/1846
Fax: +90-216-6327124

Abstract

The clinical outcome of Hepatitis B Virus (HBV) infection depends on the success or failure of the immune responses to HBV, and varies widely among individuals, ranging from asymptomatic self-limited infection, inactive carrier state, chronic hepatitis, cirrhosis, hepatocellular carcinoma, to liver failure. Genome-wide association studies (GWAS) identified key genetic factors influencing the pathogenesis of HBV-related traits. In this review, we discuss GWAS for persistence of HBV infection, antibody response to hepatitis B vaccine, and HBV-related advanced liver diseases. HBV persistence is associated with multiple genes with diverse roles in immune mechanisms. The strongest associations are found within the classical human leukocyte antigen (HLA) genes, highlighting the central role of antigen presentation in the immune response to HBV. Associated variants affect both epitope binding specificities and expression levels of HLA molecules. Several other susceptibility genes regulate the magnitude of adaptive immune responses, determining immunity *vs* tolerance. HBV persistence and nonresponse to vaccine share the same risk variants, implying overlapping genetic bases. On the other hand, the risk variants for HBV-related advanced liver diseases are largely different, suggesting different host-virus dynamics in acute *vs* chronic HBV infections. The findings of these GWAS are likely to pave the way for developing more effective preventive and therapeutic interventions by personalizing the management of HBV infection.

Key words: Genome-wide association studies; Hepatitis B infection; Hepatocellular carcinoma; Cirrhosis; Antigen

presentation; Immune response to hepatitis B virus

Core tip: Genome-wide association studies (GWAS) have proven to be very useful in uncovering the host genetic factors that influence the clinical outcomes of hepatitis B virus (HBV) infection. Both class Ⅰ and class Ⅱ human leukocyte antigen (HLA) genes were implicated in persistence of HBV infection; associated variants affected antigen-binding specificities and expression levels of HLA molecules. HBV persistence and vaccine nonresponse were associated with the same HLA-DP allotypes, suggesting a critical role for the surface antigen in HBV pathogenesis. These findings might be exploited for development of potent vaccines based on alternative epitopes. GWAS for HBV-related pathologies identified many other immune-related genes, and provided genetic markers to detect the individuals at high risk for HBV-related diseases.

INTRODUCTION

Hepatitis B virus (HBV) is the most common viral pathogen of the human liver, and is a prominent cause of acute and chronic hepatitis, liver failure, cirrhosis and hepatocellular carcinoma (HCC). Around 257 million people, or 3.5% of the global population, are estimated to have chronic HBV infection[1], and more than 800 thousands people lose their life annually due to HBV-related complications[2]. Perinatal and childhood infections are very common in regions with high endemicity, and mostly result in life-long persistence, whereas infections at adulthood are mostly self-limited[3]. Chronic HBV carriers are at a high risk of developing end-stage liver diseases, such as cirrhosis and HCC[4]. Indeed, HBV infection is responsible for 27% of cirrhosis and 53% of HCC cases worldwide[5]. Hepatitis B vaccine effectively prevents new infections, while antiviral medicines suppress progression of HBV-related liver damage. However, vaccination, safe healthcare practices, and access to treatment do not have full population coverage, and HBV infection still remains a major public health problem[6].

HBV is generally considered to be non-cytopathic *per se*; the liver injury associated with HBV infection is immune-driven. The clinical outcome of HBV infection varies greatly among individuals, ranging from asymptomatic self-limited infection, inactive carrier state, chronic hepatitis to end-stage liver diseases with life-threatening complications[3,7-9]. Viral, host and environmental factors affect these outcomes. Viral core/pre-core mutations,

certain viral genotypes (*e.g.*, genotype C in comparison to genotype B), high viral load, early-life (perinatal and childhood) infections, male sex, host genetic factors (*e.g.*, HLA class Ⅱ homozygosity), suppressed immune status, co-existing metabolic diseases, and exposure to hepatotoxic substances (*e.g.*, aflatoxin, alcohol) are associated with worse prognosis[7,10]. A complete understanding of these factors is crucial for developing more tailored and effective preventive and therapeutic interventions to reduce the burden of HBV-related complications.

The contribution of host genetic factors to the variation in HBV infection outcomes was most notably evidenced by twin studies[11], which reported higher concordance rates in monozygotic twins than in same-sex dizygotic twins for HBV carrier status[12] and for antibody titers in response to hepatitis B vaccine[13,14]. The host genetic factors were investigated in association studies whereby the frequencies of genetic variants were compared between case and control groups, using candidate gene and whole genome approaches[15-18]. Candidate gene studies focused on polymorphisms in immunologically relevant genes, especially the classical human leukocyte antigen (HLA) genes[19-21]. HLA genes encode the molecules that present antigens to T lymphocytes, and polymorphisms in these genes may alter the specificity and strength of antigen binding, affecting the T cell-mediated immune responses. In accordance with this paradigm, HLA typing studies found an ample amount of HLA allelic variations associated with the clinical outcomes of HBV infection[15]. However, the associations reported in these studies were largely inconsistent, even within the same ethnicity, with few exceptions. The validity of these studies were undermined by inappropriately small sample sizes, lack of replication in an independent cohort, ambiguous allele assignments, genotyping confined to only one or two exons that show the highest genetic variability, low population coverage, and weak statistical evidences[11,15].

The development of high-throughput genotyping technologies (*e.g.*, microarrays) and the construction of a detailed map of common genetic polymorphisms in humans enabled genome-wide investigation of genetic variants for association to complex traits and diseases. In contrast to candidate gene-based studies, genome-wide association studies (GWAS) test hundreds of thousands to millions of common SNPs across the genome, providing an unbiased method to investigate genetic risk loci, and allowing the discovery of novel disease-relevant genes. Several GWAS were conducted to identify the risk loci that predisposes to persistence of HBV infection, non-response to hepatitis B vaccine, and progression of liver disease in chronic HBV infections. In this review, we discuss the findings of these GWAS, and we emphasize how GWAS has driven the research on the genetic basis of variability in HBV-related pathologies.

GWAS FOR HBV INFECTION PERSISTENCE

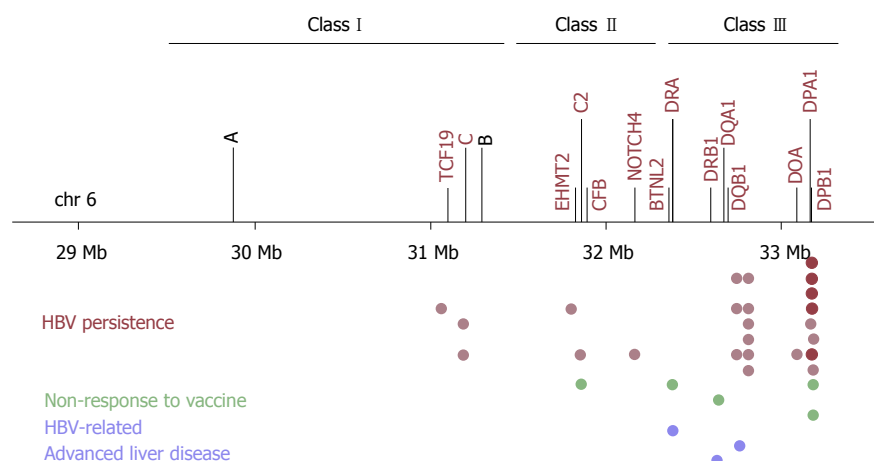The first GWAS to identify the genetic risk factors for

Figure 1 Associations within the human leukocyte antigen locus identified by genome-wide association studies for hepatitis B virus-related traits. GWAS hits for HBV-related pathologies are concentrated on the HLA region. Top SNPs in each GWAS are demonstrated in rows in the chronological order of publication; red for HBV persistence, green for Hepatitis B vaccine non-response, and blue for advanced HBV-related liver diseases. The nearest genes for the identified SNPs are colored in red; *HLA-A* and *HLA-B* are also marked. GWAS: Genome-wide association studies; HLA: Human leukocyte antigen; HBV: Hepatitis B virus; SNPs: Single nucleotide polymorphisms.

susceptibility to HBV infection persistence was performed in a Japanese population, and published in 2009[22]. Seven hundred and eighty-six chronic hepatitis B (CHB) cases and 2201 HBsAg seronegative controls were used in the discovery phase. This GWAS detected significantly associated SNPs within the *HLA-DP* locus. rs3077 in *HLA-DPA1* 3'UTR and rs9277535 in *HLA-DPB1* 3'UTR were selected, and further replicated in independent Japanese and Thai samples[22]. The same group employed a second GWAS using additional Japanese case-control samples where they confirmed the associations of *HLA-DP* variants, and, additionally, detected significant associations around the *HLA-DQ* locus[23]. The *HLA-DQ* SNPs, rs2856718 and rs7453920, were not in LD with the *HLA-DP* SNPs, and their associations remained significant after adjusting for the effect of rs9277535. These findings revealed that *HLA-DP* and *HLA-DQ* variants were independently associated with chronic HBV infection. rs2856718 tagged an LD block comprising *HLA-DQA1* and *HLA-DQB1*, which produce the functional HLA-DQ molecules. rs7453920 was located in *HLA-DQB2*, which, along with *HLA-DQA2*, is thought to be nonfunctional. The association of rs7453920 with functional *HLA-DQ* alleles was shown later[24].

Additional GWAS for HBV persistence were performed in Japanese[25], Korean[26], Han Taiwanese[27], and Han Chinese populations[28-30] (Table 1, Figure 1). These GWAS repeatedly mapped the strongest signals within the *HLA-DP* and/or *HLA-DQ* loci, replicating the associations of rs3077, rs9277535, rs7453920 and rs2856718. Furthermore, independent studies genotyping the same SNPs validated these associations with high consistency in diverse Asian populations, including Chinese[31-39], Japanese[40], Thai[41], Indonesians[42], Tibetans and Uygurs (Asian-Caucasian mix)[43]. A meta-analysis of 62050 subjects from 29 case-control studies, mostly from Asian populations, further validated the associations of rs3077 and rs9277535[44]. Replication studies were also

conducted in non-Asian populations, including Caucasians in Germany[45], European Americans, African Americans[46], Saudi Arabians population[47], and multiethnic Argentine populations (Native American-European Caucasian mix)[48]. With few exceptions and inconsistencies, probably due to different allele frequencies and LD structures in these populations, the associations of *HLA-DP* (rs3077 and rs9277535) and *HLA-DQ* (rs7453920 and rs2856718) SNPs were validated in these populations, too.

Most of the subsequent GWAS also revealed additional susceptibility loci for HBV persistence. To begin with, a GWAS in Korean chronic HBV carriers and non-infected healthy individuals identified two novel loci within the HLA region[26]. These two loci, marked by rs652888 in *EHMT2* and rs1419881 in *TCF19*, had independent effects on HBV persistence. EHMT2 is a histone lysine methyltransferase involved in gene expression regulation, and plays roles in immune cell development and differentiation[49]. TCF19 is a transcription factor necessary for cell survival and proliferation[50]. A previous GWAS associated a non-synonymous SNP in *TCF19* with blood cell counts, including lymphocyte and monocyte (macrophage precursor) cell counts[51], suggesting a potential mechanism by which *TCF19* variants affected immune mechanisms. However, the associations of rs652888 (*EHMT2*) and rs1419881 (*TCF19*) were not replicated in Chinese and Thai populations, probably due to different genetic structures[24,30,41]. Additional replication studies are, therefore, necessary for both loci.

Another GWAS in Han Chinese used chronic HBV carriers as cases and individuals who naturally cleared HBV infection as controls, and revealed two additional loci[28]. The first locus was marked by the lead SNP rs3130542, located near *HLA-C* within the HLA region. Conditional analysis showed that the effect of rs3130542 was independent of *HLA-DP* and *HLA-DQ* variants. Therefore, both class I and class II variants were detected by GWAS for HBV persistence. The second

Table 1 Results of genome-wide association studies for persistence of hepatitis B virus infection

Ref.	Study population	SNP ID	Minor/major alleles	Risk allele	P	OR	Location	Nearest gene	Functional class
Kamatani	Japanese, Thai	rs3077	A/G	G	2.31×10^{-38}	0.56	6p21.32	*HLA-DPA1*	3'UTR
et al[22]	(*n* = 6387)[1]	rs9277535	A/G	G	6.34×10^{-39}	0.57	6p21.32	*HLA-DPB1*	3'UTR
Mbarek	Japanese	rs3077	A/G	G	1.57×10^{-61}	1.87	6p21.32	*HLA-DPA1*	3'UTR
et al[23]	(*n* = 9163)[1]	rs9277535	A/G	G	2.55×10^{-54}	1.77	6p21.32	*HLA-DPB1*	3'UTR
		rs2856718	A/G	A	3.99×10^{-37}	1.56	6p21.32	*HLA-DQB1, HLA-DQA2*	Intergenic
		rs7453920	A/G	G	5.98×10^{-28}	1.81	6p21.32	*HLA-DQB2*	Intron
Nishida	Japanese	rs3077	A/G	G	4.40×10^{-19}	0.46	6p21.32	*HLA-DPA1*	3'UTR
et al[25]	(*n* = 1793)[2]	rs9277542	A/G	G	1.28×10^{-15}	0.5	6p21.32	*HLA-DPB1*	Exon
Kim	Korean	rs3077	A/G	G	3.74×10^{-40}	0.53	6p21.32	*HLA-DPA1*	3'UTR
et al[26]	(*n* = 4309)[2]	rs9277535	A/G	G	5.25×10^{-39}	0.53	6p21.32	*HLA-DPB1*	3'UTR
		rs2856718	A/G	A	1.78×10^{-24}	1.6	6p21.32	*HLA-DQB1, HLA-DQA2*	Intergenic
		rs7453920	A/G	G	6.71×10^{-26}	0.5	6p21.32	*HLA-DQB2*	Intron
		rs652888	G/A	G	7.07×10^{-13}	1.38	6p21.33	*EHMT2*	Intron
		rs1419881	G/A	A	1.26×10^{-18}	0.73	6p21.33	*TCF19*	3'UTR
Hu	Chinese	rs7453920	A/G	G	4.93×10^{-37}	0.53	6p21.32	*HLA-DQB2*	Intron
et al[28]	(*n* = 11791)[3]	rs3130542	A/G	A	9.49×10^{-14}	1.33	6p21.33	*HLA-C*	Intergenic
		rs4821116	A/G	G	1.71×10^{-12}	0.82	22q11.21	*UBE2L3*	Intron
		rs3077	A/G	G	6.50×10^{-14}	0.75	6p21.32	*HLA-DPA1*	3'UTR
Chang	Han Taiwanese	rs9277535	A/G	G	4.87×10^{-14}	1.59	6p21.32	*HLA-DPB1*	3'UTR
et al[27]	(*n* = 2688)[2]	rs7453920	A/G	G	6.66×10^{-15}	2.31	6p21.32	*HLA-DQB2*	Intron
Jiang	Chinese	rs12614	T/C	C	1.28×10^{-34}	1.89	6p21.33	*CFB*	Exon
et al[29]	(*n* = 18371)[1]	rs422951	G/A	A	5.33×10^{-16}	1.27	6p21.32	*NOTCH4*	Exon
		rs378352	T/C	T	1.04×10^{-23}	1.26	6p21.32	*HLA-DOA*	Exon
		rs2853953	A/G	G	5.06×10^{-20}	1.47	6p21.33	*HLA-C*	Intergenic
		rs1883832	T/C	T	2.95×10^{-15}	1.19	20q13.12	*CD40*	5'UTR
		rs2856718	G/A	A	7.35×10^{-28}	1.28	6p21.32	*HLA-DQB1, HLA-DQA2*	Intergenic
		rs7453920	A/G	G	1.28×10^{-60}	2	6p21.32	*HLA-DQB2*	Intron
		rs9277535	A/G	G	9.84×10^{-71}	1.52	6p21.32	*HLA-DPB1*	3'UTR
		rs3077	A/G	G	1.15×10^{-53}	1.45	6p21.32	*HLA-DPA1*	3'UTR
Li	Chinese	rs7000921	C/T	T	3.20×10^{-12}	0.78	8p21.3	*INTS10*	Intergenic
et al[30]	(*n* = 9569)[3]	rs7453920	A/G	G			6p21.32	*HLA-DQB2*	Intron
		rs9277535	A/G	G			6p21.32	*HLA-DPB1*	3'UTR

Participant phenotypes: [1]CHB *vs* Non-infected; [2]Chronic HBV carriers *vs* Non-infected; [3]Chronic HBV carriers *vs* Spontaneously recovered. OR: Odds ratio.

locus was marked by the lead SNP rs4821116, located in *UBE2L3* on chromosome 22. *UBE2L3* encodes an ubiquitin-conjugating enzyme, which enhances NFκB activation upon CD40 stimulation in B cells and TNF stimulation in monocytes[52]. The protective variant of rs4821116 was associated with higher *UBE2L3* mRNA levels in peripheral blood monocytes[53]. Moreover, *UBE2L3* was implicated in multiple autoimmune diseases; the risk variants correlated with higher *UBE2L3* expression in B cells and monocytes, enhanced NFκB activation, enhanced B cell proliferation and activation[52]. Thus, hyperactivity of UBE2L3-related immune mechanisms conferred protection from infections on the one hand, but predisposed to autoimmunity on the other hand.

The GWAS with the largest sample size (2514 chronic HBV carriers and 1130 healthy controls of Chinese ethnicity in the discovery phase) was published in 2015, revealing five new SNPs independently associated with HBV persistence[29]. Four of these SNPs, rs12614 in *CFB*, rs422951 in *NOTCH4*, rs2853953 near *HLA-C*, and rs378352 in *HLA-DO* were located within the HLA region, whereas the other SNP, rs1883832, was located in *CD40*

on chromosome 20. rs12614_T/C in *CFB* represents a non-synonymous amino acid polymorphism (R32W) in complement factor B. The complement system is part of the innate immune response against viral infections, and is also involved in enhancing adaptive immune responses[54]. Chronic HBV carriers and individuals with the risk genotype CC for rs12614 had significantly lower plasma CFB protein levels[29]. rs422951 in *NOTCH4* also causes a non-synonymous amino acid change (T320A). Notch signaling regulates immune cell development and T-cell mediated immune responses[55]. rs2853953 tagged *HLA-C* gene; however it was not in LD with the previously reported *HLA-C* tagging SNP rs3130542. Indeed, these two SNPs marked different *HLA-C* alleles; rs2853953 marked *HLA-C*06:02*, whereas rs3130542 marked *HLA-C*07:02*. rs378352 is located in exon 4 of *HLA-DOA*, causing a synonymous codon change. *HLA-DOA* encodes the α chain of the HLA-DO molecule, which, along with HLA-DM, regulates peptide loading to class Ⅱ molecules. Whether or how this polymorphism affects HLA-DO function is currently unknown. Lastly, rs1883832_T/C is located in the Kozak sequence of

Table 2 Human leukocyte antigen classical alleles associated with hepatitis B virus persistence in more than one study

HLA gene	Associated alleles	Effect on HBV persistence	Study population	Ref.
HLA-C	*07:02	Protective	Chinese	[24,28,29]
HLA-DPA1	*01:03	Protective	Japanese, Korean, Chinese, Thai	[22,24,58]
	*02:02	Susceptible	Japanese, Korean, Chinese	[22,24,58]
HLA-DPB1	*04:01	Protective	Japanese, Chinese	[22,28,58]
	*04:02	Protective	Japanese, Korean	[22,58]
	*02:01	Protective	Chinese, Japanese	[28,58]
	*05:01	Susceptible	Japanese, Korean, Chinese	[22,24,58,59]
	*09:01	Susceptible	Japanese, Chinese	[22,24,58,59]
HLA-DQA1	*06:01	Susceptible	Chinese	[24,28,30]
HLA-DQB1	*03:02	Protective	Japanese, Chinese	[24,28,30,59]
	*03:01	Susceptible	African American, Chinese	[18,28,30]
	*06:01	Susceptible	Japanese	[23,59]
HLA-DRB1	*13:02	Protective	Japanese, Chinese, Gambian, Korean, Germany, European Americans	[24,59,68]

CD40, and was previously shown to affect its translational efficiency[56]. The risk genotype TT for rs1883832 correlated with lower CD40 levels in the serum in both cases and controls[29]. CD40 is a co-stimulatory receptor protein found on antigen-presenting cells. Its engagement with CD40 ligand (CD40L) on T cells initiates and promotes humoral and cellular adaptive immune responses[57]. Intriguingly, rs1883832_T was previously associated with protection against multiple autoimmune diseases[56]. Thus, CD40 plays an important role in regulating the magnitude of immune responses, and determining immunity *vs* tolerance. So far, the associations of rs12614 (*CFB*) and rs1883832 (*CD40*) were validated by independent studies in Han Chinese[24,30]. Additional validation and functional studies are required to establish the roles of other genes and genetic variants in HBV persistence.

The most recent GWAS identified another novel locus (tagged by rs7000921) on chromosome 8 using persistently infected cases and spontaneously recovered controls in a Chinese population[30]. rs7000921 was an eQTL for the nearby *INTS10* gene; the protective allele was associated with elevated *INTS10* expression in liver. *INTS10* encodes a subunit of the Integrator complex, which has multiple roles in transcriptional regulation. Subsequent analysis showed that INTS10 was involved in suppressing HBV replication in hepatocyte cell lines, likely through an interferon-dependent mechanism[30].

To sum up, GWAS for HBV infection persistence revealed many candidate genes with diverse functions in immune responses, improving our understanding of the molecular mechanisms leading to the success or failure of HBV clearance.

CLASSICAL HLA GENE VARIANTS AT THE INTERFACE OF HBV CLEARANCE AND PERSISTENCE

GWAS association signals within the *HLA-DP*, *HLA-DQ* and *HLA-C* loci implicated variable efficiencies in presenting immunodominant HBV epitopes to T cells. Thus, in the next step after GWAS, the respective HLA genes were typed by direct sequencing or imputation (on the basis of typed SNPs from the study populations and haplotypes from the genetic reference populations), and classical HLA alleles with significant effects on HBV persistence were determined (Table 2). *HLA-DP* susceptible alleles (*DPA1*02:02*; *DPB1*05:01*, *09:01*) and protective alleles (*DPA1*01:03*; *DPB1*02:01*, *04:01*, *04:02*) were identified with a high degree of consistency in multiple Japanese, Korean and Chinese populations[22,24,28,30,58,59]. Moreover, *DPB1*01:01* and *DPB1*04:01* were identified as the most susceptible and the most protective *DPB1* allele, respectively, in European Americans and African Americans[46]. Alleles with consistent effects in more than one population were also found for *HLA-DQ* and *HLA-C* genes; *DQB1*03:02* was revealed as a protective allele[24,28,30,59], whereas *DQA1*06:01*, *DQB1*03:01*, *DQB1*06:01*, and *HLA-C*07:02* were revealed as susceptible alleles[23,24,28-30,59]. Systematic epitope discovery studies are warranted to identify the HBV-derived peptides efficiently presented by protective allotypes, but not by susceptible allotypes. The results of such studies might form the basis for epitope-specific therapeutic interventions.

A major distinction between *DPB1* protective alleles (*02:01*, *04:01*, *04:02*) and *DPB1* susceptible alleles (*01:01*, *05:01*, *09:01*) was in their amino acid residues at positions 84-87. Protective alleles encoded Gly/Gly/Pro/Met (GGPM), while susceptible alleles encoded Asn/Glu/Ala/Val (DEAV) at 84-87. These residues corresponded to the pocket-1 of the antigen-binding groove, an anchor region that determines the binding specificity of DP molecules[60,61]. Epitope binding studies revealed that DP molecules with 84GGPM87 strongly bound aromatic and hydrophobic residues in their hydrophobic pocket-1, whereas DP molecules with 84DEAV87 also preferred positively charged residues, owing to the acidic residues at 84 and 85[61,62]. These distinct binding motifs of DP allotypes likely form the basis of HBV chronicity as multiple risk-associated DP allotypes ineffectively present a common epitope motif

and lead to the failure of viral elimination. The identities of HBV peptides with this motif and their immunogenic properties remain to be determined by further functional assays.

It should be noted that the contribution of HLA-DP allotypes to HBV chronicity might also involve previously unprecedented mechanisms. It was recently shown that Gly-84 residue in DP molecules prevents the binding of the invariant chain *via* its CLIP region on the antigen-binding groove[63]. CLIP binding is necessary to block endogenous peptide binding to class Ⅱ molecules till they reach the MIIC compartment where exogenous peptide loading takes place. Therefore, DP84Gly molecules are able to present both intracellular and extracellular peptides[63]. Whether or how this contributes to immunity against HBV needs to be examined in functional studies.

The HLA locus is characterized by high gene density, high genetic diversity and extensive LD structure, limiting the ability to pinpoint the causal variants[64]. Some class Ⅱ associations might not reflect primary effects given the strong LD between *DQ* and *DR* loci, and the weak LD between *DQ-DR* and *DP* loci. Hence, uncovering the LD patterns between the *DP*, *DQ* and *DR* alleles in the study population, rather than examining the alleles at a single locus, would help detecting the alleles with primary effects[59]. Fine mapping studies in which associated loci are interrogated with many more variants than in GWAS prove to be useful in pinpointing the causal variants and revealing potential mechanisms. An interrogation of the entire HLA locus with 5375 variants, including 4356 SNPs, 849 amino acid polymorphisms and 170 classical alleles, in 1888 Chinese case-control individuals (from a previous HBV-persistence GWAS[28]) identified four independently associated loci by stepwise conditional analysis[24]. HLA-DPβ1 amino acid polymorphisms at positions 84-87 were the strongest association, whereas HLA-C Leu-15 was the second strongest association, conferring the effect of *HLA-C*07:02*. The two other associated loci were novel; *HLA-DRB*13* allele and rs400488. *HLA-DRB*13* was associated with protection against HBV chronicity, and was in partial LD with the *CFB*-tagging SNP rs12614. *HLA-DRB*13* allele is distinct from other *DRB1* alleles by the presence of Glu-71 in pocket-4, an anchor region for epitope binding of DR molecules, implying a specific binding motif. Remarkably, *HLA-DRB*13* was one of the few classical HLA alleles identified in the pre-GWAS era with highly reproducible associations across different ethnicities[65-68]. On the other hand, the biological relevance of rs400488, an eQTL for the pseudogene *HLA-J*, is currently unknown[24].

The effects of associated SNPs on HLA gene expression were also investigated. An eQTL analysis showed that the risk alleles of rs3077 and rs9277535 were associated with lower *HLA-DPA1* and *HLA-DPB1* mRNA levels, respectively, in normal liver tissues[69]. Furthermore, allelic expression imbalance assays for both SNPs confirmed that the risk alleles were expressed less abundantly than the protective alleles in liver and blood monocyte samples from heterozygous individuals[69].

Two separate genome-wide gene expression association studies previously found similar trends for the *HLA-DQ* SNP rs7453920 and the *HLA-C* SNP rs3130542. The risk variant of rs7453920 was associated with lower *HLA-DQ* mRNA levels in peripheral blood monocytes[53], while the risk variant of rs3130542 was associated with lower *HLA-C* mRNA in lymphoblastoid cell lines derived from European subjects[70]. Moreover, the susceptible *HLA-C*07:02* allele, tagged by rs3130542, have an intact mir-148a binding site, and a low expression due to RNA interference[71]. These results suggested that reduced expression of HLA-DP and HLA-DQ molecules in antigen-presenting cells, and reduced expression of HLA-C molecule in hepatocytes might result in ineffective antigen presentation to T cells, leading to inability to clear HBV infection, and thus, HBV persistence. However, a discrepant result was noted for rs9277534_A/G, the most significant *HLA-DPB1* SNP in European Americans and African Americans[46]. The risk allele rs9277534_G correlated with higher HLA-DPB1 surface protein levels in lymphoid cell lines and higher *HLA-DPB1* mRNA levels in B lymphocytes isolated from healthy donors. This conflicting result can be reconciled with the previous findings by taking into account that rs9277534_G was also in strong LD with the most susceptible *HLA-DPB1* allele (*01:01*) in these populations. Thus, the risk-conferring effect of *HLA-DPB1*01:01* probably prevailed over the protective effect of its increased expression.

To sum up, polymorphisms marking the classical HLA genes influence immune responses by altering the antigen-binding properties of HLA molecules and the expression levels thereof. The relative contributions of these effects to susceptibility to HBV persistence vary among populations depending on the allele frequencies of variants and LD relationships among them.

GWAS FOR IMMUNE RESPONSE TO HEPATITIS B VACCINE

The standard 3 doses of hepatitis B vaccine (based on recombinant HBs antigen) have been implemented as part of the routine infantile vaccination program in more than 160 countries, as a result of which substantial declines in the prevalence of chronic HBV carriers and in the incidence of childhood HBV-related liver diseases have been observed[6,72]. However, there is a large natural variation in the antibody response to hepatitis B vaccine. Post-vaccination antibody titers range from undetectable levels to higher than 2000 mIU/mL[73]. Anti-HBs titers above 100 mIU/mL are referred to as successful vaccination, whereas anti-HBs titers below 10 mIU/mL as non-protective. Individuals that mount an antibody response below this threshold, *i.e.*, non-responders, are at a high risk of HBV infection. Booster vaccination can trigger an antibody response at protective levels in a subset of non-responders[74].

Three GWAS were implemented to date to identify the genetic factors that underlie the variation in the

Table 3 Results of genome-wide association studies for non-response to hepatitis B vaccine

Ref.	Study population	SNP ID	Minor/major alleles	Risk allele	*P*	OR	Location	Nearest gene	Functional class
Png *et al*[73]	Indonesian (n = 3614)[1]	rs3135363	C/T	C	6.53×10^{-22}	1.53	6p21.32	*BTNL2, HLA-DRA*	Intergenic
		rs9277535	A/G	G	2.91×10^{-12}	0.72	6p21.32	*HLA-DPB1*	3'UTR
		rs9267665	T/C	T	1.24×10^{-17}	2.05	6p21.33	*STK19*	Intron
Pan *et al*[76]	Chinese (n = 1944)[2]	rs477515	T/C	T	2.63×10^{-19}	2.05	6p21.32	*HLA-DRB1*	Intergenic
Wu *et al*[81]	Taiwanese (n = 285)[3]	rs7770370	A/G	G	1.20×10^{-08}	0.33	6p21.32	*HLA-DPB1*	Intergenic

Participant phenotypes: [1]Low, intermediate and high responders to primary vaccine (ordinal groups); [2]High responders to primary vaccine *vs* non-responders to booster vaccine; [3]Non-responders *vs* responders to booster vaccine.

immune response to hepatitis B vaccine (Figure 1, Table 3). The first GWAS was conducted in Indonesians by grouping vaccine recipients into three classes (*i.e.*, low, intermediate and high) on the basis of their post-vaccination antibody titers[73]. Stepwise conditional analysis revealed three independently associated haplotype blocks within the HLA region. The first haplotype block, tagged by rs3135363, encompassed *HLA-DRA* and *BTNL2*. *HLA-DRA* encodes the sole α chain for HLA-DR molecules, and *BTNL2* encodes a transmembrane protein involved in the negative regulation of T cell activation. *BTNL2* was previously associated with autoimmune diseases[75]. The second haplotype block, tagged by rs9277535, encompassed *HLA-DPA1* and *HLA-DPB1* genes, suggesting that *HLA-DP* variants contribute to both HBV persistence and non-response to vaccine. A shared genetic basis for these traits is conceivable given that anti-HBs antibody production is a marker for both successful immune response to vaccine and clearance of HBV infection[3]. Lastly, the third haplotype block, tagged by rs9267665, was located within the class III region, consisting of many genes found in strong LD. Therefore, further research is needed to pinpoint the causal genes[73].

The second GWAS for vaccine nonresponse used Chinese adults with marginal phenotypes: non-responders (< 10 mIU/mL) after booster vaccination *vs* high-responders (> 1000 mIU/mL) after primary vaccination[76]. The strongest associations (led by rs477515) were detected within the *HLA-DR* locus, and *HLA-DRB1*07:01* was significantly associated with nonresponse to hepatitis B vaccination[76]. Numerous studies previously associated *DRB1*07:01* with both vaccine non-response and persistent HBV infection in multiple ethnic populations[77-79]. Furthermore, *DRB1*07* was also associated with decreased risk of HBV-related cirrhosis in a Turkish population[80], implicating a *DRB1*07*-associated hypo-immune profile against HBV.

The third GWAS used subjects with detectable and undetectable (> or < 1 mIU/mL, respectively) post-booster antibody titers from a cohort of booster vaccine recipient Taiwanese adolescents who had not responded to primary vaccination as infants[81]. Significant associations were mapped to the *HLA-DP* locus. The lead SNP rs7770370 was in strong LD with rs9277535. *HLA-DPB1* alleles *02:01*, *02:02*, *03:01*, *04:01* and *14:01* (encoding 84GGPM87) were associated with vaccine response, whereas *05:01* and *09:01* (encoding 84DEAV87) were associated with vaccine nonresponse[74,81]. These associations were further confirmed in a Japanese population[82]. Hence, *HLA-DPB1* alleles influenced the outcome of vaccine response in the same manner as of HBV persistence, corroborating further that the ability to present the same HBs epitopes is critical in both traits. In line with this, *HLA-DP* SNPs (*e.g.*, rs7770370, rs9277535 and rs3077) were also associated with vaccine nonresponse in independent replication studies in Korean infants[83] and Japanese medical students[84]; and vaccine nonresponse-associated rs477515 (*HLA-DRB1*) also correlated with HBV persistence in a Chinese population[30]. However, *HLA-DQ* SNPs (rs2856718 and rs7453920) were not associated with vaccine responsiveness[84]. Therefore, the genetic bases of HBV persistence and nonresponse to hepatitis B vaccine overlap considerably, but not completely.

GWAS FOR HBV-RELATED ADVANCED LIVER DISEASES

Host genetic variation underlying the clinical hetero-geneity of chronic HBV infections has been the topic of GWAS, too (Figure 1, Table 4). A GWAS used 648 HBV-infected Saudi Arabian subjects, comprising 343 inactive carriers, 249 patients with CHB, and 76 patients with end-stage liver diseases (*i.e.*, cirrhosis and HCC), to study liver disease progression in chronic HBV infection[85]. The strongest association, rs2724432, was obtained using cases with end-stage liver diseases and controls with inactive HBV infection. rs2724432 was located upstream of *FDX1*, the product of which is involved in electron transfer from NADH to cytochrome P450. FDX1 plays a role in steroid and vitamin D synthesis in the adrenals, and bile acid synthesis in the liver[85]. Further functional assays are necessary to confirm the association of rs2724432 and the underlying molecular mechanisms. Some of the GWAS for HBV persistence contained in their case groups chronic HBV carriers with and without

Ref.	Study population	Participant phenotypes	SNP ID	Minor/ major alleles	Risk allele	*P*	OR	Location	Nearest gene	Functional class
Al-Qahtani *et al*[85]	Saudi Arabian (*n* = 693)	LC/HCC *vs* Inactive[1]	rs2724432	T/C	T	4.29×10^{-08}	3.01	11q22.3	*FDX1*	Intergenic
Zhang *et al*[88]	Chinese (*n* = 4107)	HCC *vs* Non-HCC[2]	rs17401966	G/A	A	3.40×10^{-19}	0.62	1p36.22	*KIF1B*	Intron
Chan *et al*[91]	Chinese (*n* = 1420)	HCC *vs* Non-HCC[2]	rs12682266	A/G	G	3.76×10^{-05}	1.38	8p12	Expressed sequenced tag	Intergenic
Li *et al*[89]	Chinese (*n* = 12159)	HCC *vs* Non-HCC[2]	rs9272105	A/G	A	5.24×10^{-22}	1.28	6p21.32	*HLA-DQA1, HLA-DRB1*	Intergenic
			rs455804	A/C	C	5.24×10^{-10}	0.84	21q21.3	*GRIK1*	Intron
Jiang *et al*[90]	Chinese (*n* = 11799)	HCC *vs* Non-HCC[2]	rs7574865	T/G	G	2.48×10^{-10}	1.21	2q32.2-2q32.3	*STAT4*	Intron
			rs9275319	G/A	A	2.72×10^{-17}	1.49	6p21.32	*HLA-DQB1, HLA-DQA2*	Intergenic
Tan *et al*[86]	Chinese (*n* = 3387)	ACLF *vs* Inactive[3]	rs3129859	C/G	C	2.64×10^{-20}	1.83	6p21.32	*HLA-DQB1, HLA-DRA*	Intergenic

Table 4 Results of genome-wide association studies for hepatitis B virus-related advanced liver diseases

[1]HBV carriers with LC or HCC *vs* inactive chronic carriers; [2]Chronic HBV carriers with HCC *vs* chronic HBV carriers without HCC; [3]Acute-on-chronic liver failure *vs* inactive chronic HBV carriers. LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus.

end-stage liver diseases. At least one GWAS employed a secondary genome-wide analysis using these subgroups[25]; however, failed to identify any significant association, possibly due to small sample size.

A GWAS in Han Chinese investigated the risk loci for acute-on-chronic liver failure (ACLF) in chronic HBV carriers, a life-threatening condition that develops as a result of sudden acute exacerbation of chronic infection[86]. rs3129859 within the *HLA-DR* locus was associated with HBV-related ACLF. Subsequent in silico genotyping of *HLA* alleles and conditional association analysis showed that the effect of rs3129859 was dependent on the *HLA-DRB1*12:02* allele[86]. Concordant with this finding, *HLA-DRB1*12* was previously associated with the development of HBV-related cirrhosis and HCC in Han Chinese[87].

To find the genetic risk loci for HBV-related HCC, several GWAS were implemented using HBV carriers with HCC as cases and HCC-free HBV carriers as controls[88-92] (Table 4). All of these GWAS used Chinese cohorts, exploiting the facts that more than half of HCC cases were found in China, and 80% of HCC incidences were attributable to HBV[93]. The first GWAS identified rs17401966, located in *KIF1B* on chromosome 1[88]. *KIF1B* encodes a kinesin protein involved in organelle and vesicle transport, and is a putative tumor suppressor[94]. The protective variant of rs17401966 significantly correlated with higher KIF1B protein levels in tumor-adjacent tissues, but not in HCC tissues[88]. The haplotype block tagged by rs17401966 also encompassed the entire *PGD* gene and the 3' end of *UBE4B* gene. The products of these genes are involved in pentose phosphate metabolism and multiubiquitin chain assembly, respectively. Additional studies are necessary to identify the causal gene in this locus. Another GWAS identified rs9272105 within the HLA region and rs455804

on chromosome 21[89]. rs9272105 was located in the intergenic region between *HLA-DQA1* and *HLA-DRB1*. *HLA-DRB1* alleles *04:05* and *09:01* only partially accounted for the association of rs9272105, implying additional risk variants in LD with this SNP. rs455804 was located in intron 1 of *GRIK1*, which encodes an ionotropic glutamate receptor, suggesting involvement of glutamate signaling in hepatocarcinogenesis. Two additional risk loci were revealed in another GWAS: rs7574865 and rs9275319[90]. rs7574865 tagged *STAT4* on chromosome 2. STAT4 is a transcription factor with important roles in the regulation of antiviral immune responses; it induces IFN-γ production in response to stimulation by interleukin-12 and type I interferons (IFN-α and IFN-β)[95]. HCC risk-associated variant of rs7574865 correlated with lower *STAT4* mRNA expression in both HCC and non-tumor tissues[90]. rs9275319 tagged *HLA-DQB1* and *HLA-DQA2*, and its effect on HCC risk was only partially accounted for by *DQB1*04:01* and *DQA1*03:03* alleles, indicating other risk variants in the associated locus.

SNPs identified in GWAS for HBV-related HCC were further investigated in replication studies. Intriguingly, no evidence was found for the association of rs17401966 (*KIF1B*) with HCC risk in chronic HBV carriers in subsequent GWAS and other replication studies in Chinese[36,96], Japanese[97], Korean[97], and Thai populations[98]. Moreover, rs17401966 was also not associated with HBV infection persistence in Chinese[99], Japanese[23] and Saudi Arabian populations[100]. Comparable allele frequencies were detected between different subgroups of chronic carriers (namely, inactive carriers, active carriers, cirrhosis and HCC) in Saudi Arabian and Chinese populations[100,101]. These results indicated that rs17401966 was a risk factor neither for HBV infection persistence nor for advancement of liver disease.

Replication studies for rs7574865 (*STAT4*) produced inconsistent results. The association of rs7574865 with HBV-related HCC was validated in Vietnamese[102] and Korean[103] populations, but not in two separate Chinese populations[38,96]. The effect of rs7574865_T/G on susceptibility to persistent HBV infection was also examined. HCC risk allele rs7574865_G was significantly associated with chronic HBV infection in Koreans[103] and Chinese[104]. However, this association was not reproduced in another Chinese population[38]. Lastly, rs7574865_G was also significantly associated with the risk of HBV-related cirrhosis in a Chinese population[101]. Overall, these results supported a role for *STAT4* polymorphisms in HBV infection outcomes in a population specific manner.

The associations of the two HBV-related HCC risk SNPs within the *HLA-DQ/DR* locus (rs9275319 and rs9272105) were validated by an independent replication study in Chinese subjects[105], whereas two separate studies in Koreans[103] and in Chinese[106] failed to replicate the association of rs9275319. rs9275319 was also associated with susceptibility to HBV persistence in the original GWAS[90], and in other replication studies performed in Korean[103] and Chinese populations[30,106]. Moreover, rs9275319 was associated with increased risk of cirrhosis in Chinese HBV carriers[101]. Therefore, it might be speculated that rs9275319 was associated with an ineffective immune profile against HBV that is too weak to clear HBV infection, yet strong enough to maintain a state of basal liver necroinflammation leading to advanced liver diseases. On the other hand, the effect of rs9272105 on HBV persistence is not yet clear. HCC risk-associated variant of rs9272105 conferred susceptibility to chronic HBV infection in one Chinese population[30], and protection against chronic HBV infection in another Chinese population[89]. These conflicting results indicated that rs9272105 did not have a primary effect on HBV persistence.

To fully explore whether the genetic bases of HBV persistence and HBV-related liver diseases overlap, SNPs identified in GWAS for HBV persistence were also examined for possible effects on the development of advanced liver diseases. Most studies showed that *HLA-DP* variants (rs3077 and rs9277535) were associated neither with progression from inactive carrier state to disease-active states[35,37,39,45], nor with development of end-stage liver diseases[31,35,38,39,47]. Similar results were found for *HLA-DQ* variants (rs2856718 and rs7453920) regarding the development of end-stage liver diseases[38,39,47]. Only few studies reported significant associations of these SNPs with HBV-related HCC risk[36,89,107]. A meta-analysis with 4864 HBV-positive HCC cases and 29790 HBV-infected controls also failed to associate rs3077 and rs9277535 with HCC risk[44]. Lastly, none of the 13 HBV persistence risk SNPs that were identified hitherto in GWAS were found to be associated with HBV-related HCC in a population of 1161 cases and 1353 controls[29]. Therefore, SNPs that were associated with HBV persistence/clearance had minimal, if any, ef-

fects on progression of liver disease in chronic carriers. These results implied that distinct immune mechanisms might be critical at different stages of HBV infection, probably reflecting the evolving dynamics between HBV and the host immune system during chronic infections.

CONCLUSION

It is important to have a comprehensive understanding of the mechanisms of failure to clear HBV infection, and translate this knowledge into effective therapies to prevent the development of chronicity and other adverse outcomes. With this motive, many GWAS were performed to dissect the genetic basis of HBV-related traits. These GWAS provided novel insights into the pathogenesis of HBV persistence and non-response to hepatitis B vaccine. For instance, the association of *HLA-DPB1* with HBV persistence was unknown before GWAS. *DPB1* gene was neglected in previous candidate gene-based association studies, mainly because it was less polymorphic than *DQB1* and *DRB* genes[46]. In addition to the classical HLA genes *HLA-DP*, *HLA-DQ* and *HLA-C*, GWAS for HBV persistence provided candidate genes including non-classical HLA genes (*EHMT2*, *TCF19*, *CFB*, *NOTCH4*) and non-HLA genes (*UBE2L3*, *CD40*, *INTS10*), indicating that genetic susceptibility to HBV persistence is determined by regulation of immune responses at multiple levels. Many of these candidate genes had known roles in immune responses, whereas some were novel. Besides improving our understanding of the molecular mechanisms underlying HBV-related pathologies, GWAS findings are likely to have other immediate and long-running clinical implications. For instance, genetic markers provided by GWAS can be used to predict the individuals that are at a higher risk for worse prognosis, demanding a closer medical surveillance. Furthermore, identification of novel protective HLA allotypes might be exploited for the development of more effective vaccines based on alternative epitopes.

Despite their initial success, some of these GWAS also had certain limitations, including small sample sizes, lack of information on age at first infection, transmission route, maternal status and HBV genotype, unknown infection history of controls, unknown infection status for HCV and HIV, and imperfect match of age, gender and genetic background among some case-control groups. GWAS for HBV-related advanced liver diseases additionally suffered from the stochasticity of disease pathogenesis. For instance, the randomness of HBV DNA integration events in the host genome adds to the heterogeneity of HBV-related HCC[108]. Cirrhosis and HCC typically occur more than 20 years after the initial infection[109]. Smoking, alcohol consumption, aflatoxin exposure, obesity, diabetes, and treatment interventions during this period are strong confounders that also lead to clinical heterogeneity[110]. The lack of consistent associations in GWAS for HBV-related advanced liver diseases might be due to these confounders. Hence, these GWAS must be designed in a better way to ac-

count for each of these factors[111,112].

Interestingly, the susceptible alleles in the *HLA-DP/DQ* locus are more frequent in Asian populations than in Caucasians, which might be one of the causes for higher prevalence of chronic HBV infections in Asia[22,113]. However, most of these susceptible alleles are not frequent in Africa, too, even though chronic hepatitis B is as prevalent in Africa as in Asia. Given that the predominant mode of transmission and HBV genotypes are different in Asia and Africa (vertical *vs* horizontal; B, C *vs* A, D, E; respectively), the genetic architecture for predisposition to chronic HBV infection also varies among these populations[7,113]. Hence, allele frequency distributions across human populations might give important insights into human-HBV co-evolution.

GWAS for HBV-related traits have almost exclusively been implemented in Asian populations. So far, there is no GWAS in Africans, and only one GWAS in Saudi Arabians, where the dominant HBV genotype is D[7]. Populations with different ancestries harbor different haplotype structures and allele frequencies; therefore, implementing GWAS in other populations might also help narrow down the associated loci. This is especially desirable for fine mapping studies. Fine mapping studies on the HLA locus, where extensive LD structure hampers the detection of causal variants, are of utmost importance since immune-related genes are concentrated in this region. The findings of such studies would reveal novel insights into the pathogenesis of HBV-related traits. Therefore, additional GWAS and fine mapping studies, implemented with more refined case-control designs, larger samples, and in other ethnic populations, would further improve our understanding of HBV infection pathophysiology.

REFERENCES

1   **Schweitzer A**, Horn J, Mikolajczyk RT, Krause G, Ott JJ. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. *Lancet* 2015; **386**: 1546-1555 [PMID: 26231459 DOI: 10.1016/S0140-6736(15)61412-X]

2   **Lozano R**, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn SY, Alvarado M, Anderson HR, Anderson LM, Andrews KG, Atkinson C, Baddour LM, Barker-Collo S, Bartels DH, Bell ML, Benjamin EJ, Bennett D, Bhalla K, Bikbov B, Bin Abdulhak A, Birbeck G, Blyth F, Bolliger I, Boufous S, Bucello C, Burch M, Burney P, Carapetis J, Chen H, Chou D, Chugh SS, Coffeng LE, Colan SD, Colquhoun S, Colson KE, Condon J, Connor MD, Cooper LT, Corriere M, Cortinovis M, de Vaccaro KC, Couser W, Cowie BC, Criqui MH, Cross M, Dabhadkar KC, Dahodwala N, De Leo D, Degenhardt L, Delossantos A, Denenberg J, Des Jarlais DC, Dharmaratne SD, Dorsey ER, Driscoll T, Duber H, Ebel B, Erwin PJ, Espindola P, Ezzati M, Feigin V, Flaxman AD, Forouzanfar MH, Fowkes FG, Franklin R, Fransen M, Freeman MK, Gabriel SE, Gakidou E, Gaspari F, Gillum RF, Gonzalez-Medina D, Halasa YA, Haring D, Harrison JE, Havmoeller R, Hay RJ, Hoen B, Hotez PJ, Hoy D, Jacobsen KH, James SL, Jasrasaria R, Jayaraman S, Johns N, Karthikeyan G, Kassebaum N, Keren A, Khoo JP, Knowlton LM, Kobusingye O, Koranteng A, Krishnamurthi R, Lipnick M, Lipshultz SE, Ohno SL, Mabweijano J, MacIntyre MF, Mallinger L, March L, Marks GB, Marks R, Matsumori A, Matzopoulos R, Mayosi BM, McAnulty JH, McDermott MM, McGrath J, Mensah GA, Merriman TR, Michaud C, Miller M, Miller TR, Mock C, Mocumbi AO, Mokdad AA, Moran A, Mulholland K, Nair MN, Naldi L, Narayan KM, Nasseri K, Norman P, O'Donnell M, Omer SB, Ortblad K, Osborne R, Ozgediz D, Pahari B, Pandian JD, Rivero AP, Padilla RP, Perez-Ruiz F, Perico N, Phillips D, Pierce K, Pope CA 3rd, Porrini E, Pourmalek F, Raju M, Ranganathan D, Rehm JT, Rein DB, Remuzzi G, Rivara FP, Roberts T, De León FR, Rosenfeld LC, Rushton L, Sacco RL, Salomon JA, Sampson U, Sanman E, Schwebel DC, Segui-Gomez M, Shepard DS, Singh D, Singleton J, Sliwa K, Smith E, Steer A, Taylor JA, Thomas B, Tleyjeh IM, Towbin JA, Truelsen T, Undurraga EA, Venketasubramanian N, Vijayakumar L, Vos T, Wagner GR, Wang M, Wang W, Watt K, Weinstock MA, Weintraub R, Wilkinson JD, Woolf AD, Wulf S, Yeh PH, Yip P, Zabetian A, Zheng ZJ, Lopez AD, Murray CJ, AlMazroa MA, Memish ZA. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012; **380**: 2095-2128 [PMID: 23245604 DOI: 10.1016/S0140-6736(12)61728-0]

3   **Trépo C**, Chan HL, Lok A. Hepatitis B virus infection. *Lancet* 2014; **384**: 2053-2063 [PMID: 24954675 DOI: 10.1016/S0140-6736(14)60220-8]

4   **Beasley RP**, Hwang LY, Lin CC, Chien CS. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22 707 men in Taiwan. *Lancet* 1981; **2**: 1129-1133 [PMID: 6118576 DOI: 10.1016/S0140-6736(81)90585-7]

5   **Perz JF**, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol* 2006; **45**: 529-538 [PMID: 16879891 DOI: 10.1016/j.jhep.2006.05.013]

6   **World Health Organization**. Global Hepatitis Report 2017. Accessed October 2017. [ISBN: 978-92-4-156545-5]. Available from: URL: http://www.who.int/hepatitis/publications/global-hepatitis-report2017/en/

7   **McMahon BJ**. The natural history of chronic hepatitis B virus infection. *Hepatology* 2009; **49**: S45-S55 [PMID: 19399792 DOI: 10.1002/hep.22898]

8   **Liang TJ**. Hepatitis B: the virus and disease. *Hepatology* 2009; **49**: S13-S21 [PMID: 19399811 DOI: 10.1002/hep.22881]

9   **Hoofnagle JH**, Doo E, Liang TJ, Fleischer R, Lok AS. Management of hepatitis B: summary of a clinical research workshop. *Hepatology* 2007; **45**: 1056-1075 [PMID: 17393513 DOI: 10.1002/hep.21627]

10   **Nguyen VT**, Law MG, Dore GJ. Hepatitis B-related hepatocellular carcinoma: epidemiological characteristics and disease burden. *J Viral Hepat* 2009; **16**: 453-463 [PMID: 19302335 DOI: 10.1111/j.1365-2893.2009.01117.x]

11   **He YL**, Zhao YR, Zhang SL, Lin SM. Host susceptibility to persistent hepatitis B virus infection. *World J Gastroenterol* 2006; **12**: 4788-4793 [PMID: 16937459 DOI: 10.3748/WJG.V12.I30.4788]

12   **Lin TM**, Chen CJ, Wu MM, Yang CS, Chen JS, Lin CC, Kwang TY, Hsu ST, Lin SY, Hsu LC. Hepatitis B virus markers in Chinese twins. *Anticancer Res* 1989; **9**: 737-741 [PMID: 2764519]

13   **Höhler T**, Reuss E, Evers N, Dietrich E, Rittner C, Freitag CM, Vollmar J, Schneider PM, Fimmers R. Differential genetic determination of immune responsiveness to hepatitis B surface antigen and to hepatitis A virus: a vaccination study in twins. *Lancet* 2002; **360**: 991-995 [PMID: 12383669 DOI: 10.1016/S0140-6736(02)11083-X]

14   **Newport MJ**, Goetghebuer T, Weiss HA, Whittle H, Siegrist CA, Marchant A; MRC Gambia Twin Study Group. Genetic regulation of immune responses to vaccines in early life. *Genes Immun* 2004; **5**: 122-129 [PMID: 14737096 DOI: 10.1038/sj.gene.6364051]

15   **Singh R**, Kaul R, Kaul A, Khan K. A comparative review of HLA associations with hepatitis B and C viral infections across global populations. *World J Gastroenterol* 2007; **13**: 1770-1787 [PMID: 17465466 DOI: 10.3748/wjg.v13.i12.1770]

16   **Wang L**, Zou ZQ, Wang K. Clinical Relevance of HLA Gene Variants in HBV Infection. *J Immunol Res* 2016; **2016**: 9069375 [PMID: 27243039 DOI: 10.1155/2016/9069375]

17   **Matsuura K**, Isogawa M, Tanaka Y. Host genetic variants influencing the clinical course of hepatitis B virus infection. *J Med Virol* 2016; **88**: 371-379 [PMID: 26255971 DOI: 10.1002/jmv.24350]

18   **Tong Hv**, Bock CT, Velavan TP. Genetic insights on host and hepatitis B virus in liver diseases. *Mutat Res Rev Mutat Res* 2014; **762**: 65-75 [PMID: 25475418 DOI: 10.1016/j.mrrev.2014.06.001]

19   **Katrinli S**, Nigdelioglu A, Ozdil K, Dinler-Doganay G, Doganay L. The association of variations in TLR genes and spontaneous immune control of hepatitis B virus. *Clin Res Hepatol Gastroenterol* 2018; **42**: 139-144 [PMID: 29276096 DOI: 10.1016/j.clinre.2017.10.002]

20   **Katrinli S**, Enc FY, Ozdil K, Ozturk O, Tuncer I, Doganay GD, Doganay L. Effect of HLA-DPA1 alleles on chronic hepatitis B prognosis and treatment response. *North Clin Istanb* 2017; **3**: 168-174 [PMID: 28275747 DOI: 10.14744/nci.2016.27870]

21   **Doganay L**, Tuncer I, Katrinli S, Enc FY, Ozturk O, Colak Y, Ulasoglu C, Dinler G. The effect of HLA-DQB1 alleles on virologic breakthroughs during chronic hepatitis B treatment with genetically low barrier drugs. *Clin Res Hepatol Gastroenterol* 2013; **37**: 359-364 [PMID: 23273495 DOI: 10.1016/j.clinre.2012.10.013]

22   **Kamatani Y**, Wattanapokayakit S, Ochi H, Kawaguchi T, Takahashi A, Hosono N, Kubo M, Tsunoda T, Kamatani N, Kumada H, Puseenam A, Sura T, Daigo Y, Chayama K, Chantratita W, Nakamura Y, Matsuda K. A genome-wide association study identifies variants in the HLA-DP locus associated with chronic hepatitis B in Asians. *Nat Genet* 2009; **41**: 591-595 [PMID: 19349983 DOI: 10.1038/ng.348]

23   **Mbarek H**, Ochi H, Urabe Y, Kumar V, Kubo M, Hosono N, Takahashi A, Kamatani Y, Miki D, Abe H, Tsunoda T, Kamatani N, Chayama K, Nakamura Y, Matsuda K. A genome-wide association study of chronic hepatitis B identified novel risk locus in a Japanese population. *Hum Mol Genet* 2011; **20**: 3884-3892 [PMID: 21750111 DOI: 10.1093/hmg/ddr301]

24   **Zhu M**, Dai J, Wang C, Wang Y, Qin N, Ma H, Song C, Zhai X, Yang Y, Liu J, Liu L, Li S, Liu J, Yang H, Zhu F, Shi Y, Shen H, Jin G, Zhou W, Hu Z. Fine mapping the MHC region identified four independent variants modifying susceptibility to chronic hepatitis B in Han Chinese. *Hum Mol Genet* 2016; **25**: 1225-1232 [PMID: 26769676 DOI: 10.1093/hmg/ddw003]

25   **Nishida N**, Sawai H, Matsuura K, Sugiyama M, Ahn SH, Park JY, Hige S, Kang JH, Suzuki K, Kuroaki M, Asahina Y, Mochida S, Watanabe M, Tanaka E, Honda M, Kaneko S, Orito E, Itoh Y, Mita E, Tamori A, Murawaki Y, Hiasa Y, Sakaida I, Korenaga M, Hino K, Ide T, Kawashima M, Mawatari Y, Sageshima M, Ogasawara Y, Koike A, Izumi N, Han KH, Tanaka Y, Tokunaga K, Mizokami M. Genome-wide association study confirming association of HLA-DP with protection against chronic hepatitis B and viral clearance in Japanese and Korean. *PLoS One* 2012; **7**: e39175 [PMID: 22737229 DOI: 10.1371/journal.pone.0039175]

26   **Kim YJ**, Kim HY, Lee JH, Yu SJ, Yoon JH, Lee HS, Kim CY, Cheong JY, Cho SW, Park NH, Park BL, Namgoong S, Kim LH, Cheong HS, Shin HD. A genome-wide association study identified new variants associated with the risk of chronic hepatitis B. *Hum Mol Genet* 2013; **22**: 4233-4238 [PMID: 23760081 DOI: 10.1093/hmg/ddt266]

27   **Chang SW**, Fann CS, Su WH, Wang YC, Weng CC, Yu CJ, Hsu CL, Hsieh AR, Chien RN, Chu CM, Tai DI. A genome-wide association study on chronic HBV infection and its clinical progression in male Han-Taiwanese. *PLoS One* 2014; **9**: e99724 [PMID: 24940741 DOI: 10.1371/journal.pone.0099724]

28   **Hu Z**, Liu Y, Zhai X, Dai J, Jin G, Wang L, Zhu L, Yang Y, Liu J, Chu M, Wen J, Xie K, Du G, Wang Q, Zhou Y, Cao M, Liu L, He Y, Wang Y, Zhou G, Jia W, Lu J, Li S, Liu J, Yang H, Shi Y, Zhou W, Shen H. New loci associated with chronic hepatitis B virus infection in Han Chinese. *Nat Genet* 2013; **45**: 1499-1503 [PMID: 24162738 DOI: 10.1038/ng.2809]

29   **Jiang DK**, Ma XP, Yu H, Cao G, Ding DL, Chen H, Huang HX, Gao YZ, Wu XP, Long XD, Zhang H, Zhang Y, Gao Y, Chen TY, Ren WH, Zhang P, Shi Z, Jiang W, Wan B, Saiyin H, Yin J, Zhou YF, Zhai Y, Lu PX, Zhang H, Gu X, Tan A, Wang JB, Zuo XB, Sun LD, Liu JO, Yi Q, Mo Z, Zhou G, Liu Y, Sun J, Shugart YY, Zheng SL,

Zhang XJ, Xu J, Yu L. Genetic variants in five novel loci including CFB and CD40 predispose to chronic hepatitis B. *Hepatology* 2015; **62**: 118-128 [PMID: 25802187 DOI: 10.1002/hep.27794]

30   **Li Y**, Si L, Zhai Y, Hu Y, Hu Z, Bei JX, Xie B, Ren Q, Cao P, Yang F, Song Q, Bao Z, Zhang H, Han Y, Wang Z, Chen X, Xia X, Yan H, Wang R, Zhang Y, Gao C, Meng J, Tu X, Liang X, Cui Y, Liu Y, Wu X, Li Z, Wang H, Li Z, Hu B, He M, Gao Z, Xu X, Ji H, Yu C, Sun Y, Xing B, Yang X, Zhang H, Tan A, Wu C, Jia W, Li S, Zeng YX, Shen H, He F, Mo Z, Zhang H, Zhou G. Genome-wide association study identifies 8p21.3 associated with persistent hepatitis B virus infection among Chinese. *Nat Commun* 2016; **7**: 11664 [PMID: 27244555 DOI: 10.1038/ncomms11664]

31   **An P**, Winkler C, Guan L, O'Brien SJ, Zeng Z; HBV Study Consortium. A common HLA-DPA1 variant is a major determinant of hepatitis B virus clearance in Han Chinese. *J Infect Dis* 2011; **203**: 943-947 [PMID: 21402545 DOI: 10.1093/infdis/jiq154]

32   **Lau KC**, Lam CW, Law CY, Lai ST, Tsang TY, Siu CW, To WK, Leung KF, Mak CM, Poon WT, Chan PK, Chan YW. Non-invasive screening of HLA-DPA1 and HLA-DPB1 alleles for persistent hepatitis B virus infection: susceptibility for vertical transmission and toward a personalized approach for vaccination and treatment. *Clin Chim Acta* 2011; **412**: 952-957 [PMID: 21310144 DOI: 10.1016/j.cca.2011.01.030]

33   **Wang L**, Wu XP, Zhang W, Zhu DH, Wang Y, Li YP, Tian Y, Li RC, Li Z, Zhu X, Li JH, Cai J, Liu L, Miao XP, Liu Y, Li H. Evaluation of genetic susceptibility loci for chronic hepatitis B in Chinese: two independent case-control studies. *PLoS One* 2011; **6**: e17608 [PMID: 21408128 DOI: 10.1371/journal.pone.0017608]

34   **Guo X**, Zhang Y, Li J, Ma J, Wei Z, Tan W, O'Brien SJ. Strong influence of human leukocyte antigen (HLA)-DP gene variants on development of persistent chronic hepatitis B virus carriers in the Han Chinese population. *Hepatology* 2011; **53**: 422-428 [PMID: 21274863 DOI: 10.1002/hep.24048]

35   **Li J**, Yang D, He Y, Wang M, Wen Z, Liu L, Yao J, Matsuda K, Nakamura Y, Yu J, Jiang X, Sun S, Liu Q, Jiang X, Song Q, Chen M, Yang H, Tang F, Hu X, Wang J, Chang Y, He X, Chen Y, Lin J. Associations of HLA-DP variants with hepatitis B virus infection in southern and northern Han Chinese populations: a multicenter case-control study. *PLoS One* 2011; **6**: e24221 [PMID: 21904616 DOI: 10.1371/journal.pone.0024221]

36   **Hu L**, Zhai X, Liu J, Chu M, Pan S, Jiang J, Zhang Y, Wang H, Chen J, Shen H, Hu Z. Genetic variants in human leukocyte antigen/DP-DQ influence both hepatitis B virus clearance and hepatocellular carcinoma development. *Hepatology* 2012; **55**: 1426-1431 [PMID: 22105689 DOI: 10.1002/hep.24799]

37   **Wong DK**, Watanabe T, Tanaka Y, Seto WK, Lee CK, Fung J, Lin CK, Huang FY, Lai CL, Yuen MF. Role of HLA-DP polymorphisms on chronicity and disease activity of hepatitis B infection in Southern Chinese. *PLoS One* 2013; **8**: e66920 [PMID: 23825586 DOI: 10.1371/journal.pone.0066920]

38   **Liao Y**, Cai B, Li Y, Chen J, Tao C, Huang H, Wang L. Association of HLA-DP/DQ and STAT4 polymorphisms with HBV infection outcomes and a mini meta-analysis. *PLoS One* 2014; **9**: e111677 [PMID: 25365208 DOI: 10.1371/journal.pone.0111677]

39   **Tao J**, Su K, Yu C, Liu X, Wu W, Xu W, Jiang B, Luo R, Yao J, Zhou J, Zhan Y, Ye C, Yuan W, Jiang X, Cui W, Li MD, Li L. Fine mapping analysis of HLA-DP/DQ gene clusters on chromosome 6 reveals multiple susceptibility loci for HBV infection. *Amino Acids* 2015; **47**: 2623-2634 [PMID: 26197724 DOI: 10.1007/s00726-015-2054-6]

40   **Zhang X**, Jia J, Dong J, Yu F, Ma N, Li M, Liu X, Liu W, Li T, Liu D. HLA-DQ polymorphisms with HBV infection: different outcomes upon infection and prognosis to lamivudine therapy. *J Viral Hepat* 2014; **21**: 491-498 [PMID: 24750255 DOI: 10.1111/jvh.12159]

41   **Posuwan N**, Payungporn S, Tangkijvanich P, Ogawa S, Murakami S, Iijima S, Matsuura K, Shinkai N, Watanabe T, Poovorawan Y, Tanaka Y. Genetic association of human leukocyte antigens with chronicity or resolution of hepatitis B infection in thai population. *PLoS One* 2014; **9**: e86007 [PMID: 24465836 DOI: 10.1371/journal.pone.0086007]

42   **Wasityastuti W**, Yano Y, Ratnasari N, Triyono T, Triwikatmani C, Indrarti F, Heriyanto DS, Yamani LN, Liang Y, Utsumi T, Hayashi Y. Protective effects of HLA-DPA1/DPB1 variants against Hepatitis B virus infection in an Indonesian population. *Infect Genet Evol* 2016; **41**: 177-184 [PMID: 27051043 DOI: 10.1016/j.meegid.2016.03.034]

43   **Liao Y**, Cai B, Li Y, Chen J, Ying B, Tao C, Zhao M, Ba Z, Zhang Z, Wang L. Association of HLA-DP/DQ, STAT4 and IL-28B variants with HBV viral clearance in Tibetans and Uygurs in China. *Liver Int* 2015; **35**: 886-896 [PMID: 25041342 DOI: 10.1111/liv.12643]

44   **Yu L**, Cheng YJ, Cheng ML, Yao YM, Zhang Q, Zhao XK, Liu HJ, Hu YX, Mu M, Wang B, Yang GZ, Zhu LL, Zhang S. Quantitative assessment of common genetic variations in HLA-DP with hepatitis B virus infection, clearance and hepatocellular carcinoma development. *Sci Rep* 2015; **5**: 14933 [PMID: 26462556 DOI: 10.1038/srep14933]

45   **Vermehren J**, Lötsch J, Susser S, Wicker S, Berger A, Zeuzem S, Sarrazin C, Doehring A. A common HLA-DPA1 variant is associated with hepatitis B virus infection but fails to distinguish active from inactive Caucasian carriers. *PLoS One* 2012; **7**: e32605 [PMID: 22448225 DOI: 10.1371/journal.pone.0032605]

46   **Thomas R**, Thio CL, Apps R, Qi Y, Gao X, Marti D, Stein JL, Soderberg KA, Moody MA, Goedert JJ, Kirk GD, Hoots WK, Wolinsky S, Carrington M. A novel variant marking HLA-DP expression levels predicts recovery from hepatitis B virus infection. *J Virol* 2012; **86**: 6979-6985 [PMID: 22496224 DOI: 10.1128/JVI.00406-12]

47   **Al-Qahtani AA**, Al-Anazi MR, Abdo AA, Sanai FM, Al-Hamoudi W, Alswat KA, Al-Ashgar HI, Khalaf NZ, Eldali AM, Viswan NA, Al-Ahdal MN. Association between HLA variations and chronic hepatitis B virus infection in Saudi Arabian patients. *PLoS One* 2014; **9**: e80445 [PMID: 24465366 DOI: 10.1371/journal.pone.0080445]

48   **Trinks J**, Nishida N, Hulaniuk ML, Caputo M, Tsuchiura T, Marciano S, Haddad L, Blejer J, Bartoli S, Ameigeiras B, Frías SE, Vistarini C, Heinrich F, Remondegui C, Ceballos S, Echenique G, Charre Samman M, D'Amico C, Rojas A, Martínez A, Ridruejo E, Fernández RJ, Burgos Pratx L, Salamone H, Nuñez F, Galdame O, Gadano A, Corach D, Sugiyama M, Flichman D, Tokunaga K, Mizokami M. Role of HLA-DP and HLA-DQ on the clearance of hepatitis B virus and the risk of chronic infection in a multiethnic population. *Liver Int* 2017; **37**: 1476-1487 [PMID: 28267888 DOI: 10.1111/liv.13405]

49   **Scheer S**, Zaph C. The Lysine Methyltransferase G9a in Immune Cell Differentiation and Function. *Front Immunol* 2017; **8**: 429 [PMID: 28443098 DOI: 10.3389/fimmu.2017.00429]

50   **Krautkramer KA**, Linnemann AK, Fontaine DA, Whillock AL, Harris TW, Schleis GJ, Truchan NA, Marty-Santos L, Lavine JA, Cleaver O, Kimple ME, Davis DB. Tcf19 is a novel islet factor necessary for proliferation and survival in the INS-1 β-cell line. *Am J Physiol Endocrinol Metab* 2013; **305**: E600-E610 [PMID: 23860123 DOI: 10.1152/ajpendo.00147.2013]

51   **Ferreira MA**, Hottenga JJ, Warrington NM, Medland SE, Willemsen G, Lawrence RW, Gordon S, de Geus EJ, Henders AK, Smit JH, Campbell MJ, Wallace L, Evans DM, Wright MJ, Nyholt DR, James AL, Beilby JP, Penninx BW, Palmer LJ, Frazer IH, Montgomery GW, Martin NG, Boomsma DI. Sequence variants in three loci influence monocyte counts and erythrocyte volume. *Am J Hum Genet* 2009; **85**: 745-749 [PMID: 19853236 DOI: 10.1016/j.ajhg.2009.10.005]

52   **Lewis MJ**, Vyse S, Shields AM, Boeltz S, Gordon PA, Spector TD, Lehner PJ, Walczak H, Vyse TJ. UBE2L3 polymorphism amplifies NF-κB activation and promotes plasma cell development, linking linear ubiquitination to multiple autoimmune diseases. *Am J Hum Genet* 2015; **96**: 221-234 [PMID: 25640675 DOI: 10.1016/j.ajhg.2014.12.024]

53   **Zeller T**, Wild P, Szymczak S, Rotival M, Schillert A, Castagne R, Maouche S, Germain M, Lackner K, Rossmann H, Eleftheriadis M, Sinning CR, Schnabel RB, Lubos E, Mennerich D, Rust W, Perret C, Proust C, Nicaud V, Loscalzo J, Hübner N, Tregouet D, Münzel T, Ziegler A, Tiret L, Blankenberg S, Cambien F. Genetics and beyond-the transcriptome of human monocytes and disease susceptibility.

PLoS One 2010; **5**: e10693 [PMID: 20502693 DOI: 10.1371/journal.pone.0010693]

54   **Stoermer KA**, Morrison TE. Complement and viral pathogenesis. *Virology* 2011; **411**: 362-373 [PMID: 21292294 DOI: 10.1016/j.virol.2010.12.045]

55   **Radtke F**, Fasnacht N, Macdonald HR. Notch signaling in the immune system. *Immunity* 2010; **32**: 14-27 [PMID: 20152168 DOI: 10.1016/j.immuni.2010.01.004]

56   **Jacobson EM**, Concepcion E, Oashi T, Tomer Y. A Graves' disease-associated Kozak sequence single-nucleotide polymorphism enhances the efficiency of CD40 gene translation: a case for translational pathophysiology. *Endocrinology* 2005; **146**: 2684-2691 [PMID: 15731360 DOI: 10.1210/en.2004-1617]

57   **Elgueta R**, Benson MJ, de Vries VC, Wasiuk A, Guo Y, Noelle RJ. Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol Rev* 2009; **229**: 152-172 [PMID: 19426221 DOI: 10.1111/j.1600-065X.2009.00782.x]

58   **Nishida N**, Sawai H, Kashiwase K, Minami M, Sugiyama M, Seto WK, Yuen MF, Posuwan N, Poovorawan Y, Ahn SH, Han KH, Matsuura K, Tanaka Y, Kurosaki M, Asahina Y, Izumi N, Kang JH, Hige S, Ide T, Yamamoto K, Sakaida I, Murawaki Y, Itoh Y, Tamori A, Orito E, Hiasa Y, Honda M, Kaneko S, Mita E, Suzuki K, Hino K, Tanaka E, Mochida S, Watanabe M, Eguchi Y, Masaki N, Murata K, Korenaga M, Mawatari Y, Ohashi J, Kawashima M, Tokunaga K, Mizokami M. New susceptibility and resistance HLA-DP alleles to HBV-related diseases identified by a trans-ethnic association study in Asia. *PLoS One* 2014; **9**: e86449 [PMID: 24520320 DOI: 10.1371/journal.pone.0086449]

59   **Nishida N**, Ohashi J, Khor SS, Sugiyama M, Tsuchiura T, Sawai H, Hino K, Honda M, Kaneko S, Yatsuhashi H, Yokosuka O, Koike K, Kurosaki M, Izumi N, Korenaga M, Kang JH, Tanaka E, Taketomi A, Eguchi Y, Sakamoto N, Yamamoto K, Tamori A, Sakaida I, Hige S, Itoh Y, Mochida S, Mita E, Takikawa Y, Ide T, Hiasa Y, Kojima H, Yamamoto K, Nakamura M, Saji H, Sasazuki T, Kanto T, Tokunaga K, Mizokami M. Understanding of HLA-conferred susceptibility to chronic hepatitis B infection requires HLA genotyping-based association analysis. *Sci Rep* 2016; **6**: 24767 [PMID: 27091392 DOI: 10.1038/srep24767]

60   **Díaz G**, Amicosante M, Jaraquemada D, Butler RH, Guillén MV, Sánchez M, Nombela C, Arroyo J. Functional analysis of HLA-DP polymorphism: a crucial role for DPbeta residues 9, 11, 35, 55, 56, 69 and 84-87 in T cell allorecognition and peptide binding. *Int Immunol* 2003; **15**: 565-576 [PMID: 12697658 DOI: 10.1093/intimm/dxg057]

61   **Sidney J**, Steen A, Moore C, Ngo S, Chung J, Peters B, Sette A. Five HLA-DP molecules frequently expressed in the worldwide human population share a common HLA supertypic binding specificity. *J Immunol* 2010; **184**: 2492-2503 [PMID: 20139279 DOI: 10.4049/jimmunol.0903655]

62   **Andreatta M**, Nielsen M. Characterizing the binding motifs of 11 common human HLA-DP and HLA-DQ molecules using NNAlign. *Immunology* 2012; **136**: 306-311 [PMID: 22352343 DOI: 10.1111/j.1365-2567.2012.03579.x]

63   **Yamashita Y**, Anczurowski M, Nakatsugawa M, Tanaka M, Kagoya Y, Sinha A, Chamoto K, Ochi T, Guo T, Saso K, Butler MO, Minden MD, Kislinger T, Hirano N. HLA-DP[84Gly] constitutively presents endogenous peptides generated by the class I antigen processing pathway. *Nat Commun* 2017; **8**: 15244 [PMID: 28489076 DOI: 10.1038/ncomms15244]

64   **de Bakker PI**, Raychaudhuri S. Interrogating the major histocompatibility complex with high-throughput genomics. *Hum Mol Genet* 2012; **21**: R29-R36 [PMID: 22976473 DOI: 10.1093/hmg/dds384]

65   **Thursz MR**, Kwiatkowski D, Allsopp CE, Greenwood BM, Thomas HC, Hill AV. Association between an MHC class Ⅱ allele and clearance of hepatitis B virus in the Gambia. *N Engl J Med* 1995; **332**: 1065-1069 [PMID: 7898524 DOI: 10.1056/NEJM199504203321604]

66   **Höhler T**, Gerken G, Notghi A, Lubjuhn R, Taheri H, Protzer U, Löhr HF, Schneider PM, Meyer zum Büschenfelde KH, Rittner C.

HLA-DRB1*1301 and *1302 protect against chronic hepatitis B. *J Hepatol* 1997; **26**: 503-507 [PMID: 9075656 DOI: 10.1016/S0168-8278(97)80414-X]

67   **Ahn SH**, Han KH, Park JY, Lee CK, Kang SW, Chon CY, Kim YS, Park K, Kim DK, Moon YM. Association between hepatitis B virus infection and HLA-DR type in Korea. *Hepatology* 2000; **31**: 1371-1373 [PMID: 10827165 DOI: 10.1053/jhep.2000.7988]

68   **Thio CL**, Thomas DL, Karacki P, Gao X, Marti D, Kaslow RA, Goedert JJ, Hilgartner M, Strathdee SA, Duggal P, O'Brien SJ, Astemborski J, Carrington M. Comprehensive analysis of class I and class Ⅱ HLA antigens and chronic hepatitis B virus infection. *J Virol* 2003; **77**: 12083-12087 [PMID: 14581545 DOI: 10.1128/jvi.77.22.12083-12087.2003]

69   **O'Brien TR**, Kohaar I, Pfeiffer RM, Maeder D, Yeager M, Schadt EE, Prokunina-Olsson L. Risk alleles for chronic hepatitis B are associated with decreased mRNA expression of HLA-DPA1 and HLA-DPB1 in normal human liver. *Genes Immun* 2011; **12**: 428-433 [PMID: 21346778 DOI: 10.1038/gene.2011.11]

70   **Montgomery SB**, Sammeth M, Gutierrez-Arcelus M, Lach RP, Ingle C, Nisbett J, Guigo R, Dermitzakis ET. Transcriptome genetics using second generation sequencing in a Caucasian population. *Nature* 2010; **464**: 773-777 [PMID: 20220756 DOI: 10.1038/nature08903]

71   **Kaur G**, Gras S, Mobbs JI, Vivian JP, Cortes A, Barber T, Kuttikkatte SB, Jensen LT, Attfield KE, Dendrou CA, Carrington M, McVean G, Purcell AW, Rossjohn J, Fugger L. Structural and regulatory diversity shape HLA-C protein expression levels. *Nat Commun* 2017; **8**: 15924 [PMID: 28649982 DOI: 10.1038/ncomms15924]

72   **Zanetti AR**, Van Damme P, Shouval D. The global impact of vaccination against hepatitis B: a historical overview. *Vaccine* 2008; **26**: 6266-6273 [PMID: 18848855 DOI: 10.1016/j.vaccine.2008.09.056]

73   **Png E**, Thalamuthu A, Ong RT, Snippe H, Boland GJ, Seielstad M. A genome-wide association study of hepatitis B vaccine response in an Indonesian population reveals multiple independent risk variants in the HLA region. *Hum Mol Genet* 2011; **20**: 3893-3898 [PMID: 21764829 DOI: 10.1093/hmg/ddr302]

74   **Wu TW**, Chu CC, Ho TY, Chang Liao HW, Lin SK, Lin M, Lin HH, Wang LY. Responses to booster hepatitis B vaccination are significantly correlated with genotypes of human leukocyte antigen (HLA)-DPB1 in neonatally vaccinated adolescents. *Hum Genet* 2013; **132**: 1131-1139 [PMID: 23739870 DOI: 10.1007/s00439-013-1320-5]

75   **Nguyen T**, Liu XK, Zhang Y, Dong C. BTNL2, a butyrophilin-like molecule that functions to inhibit T cell activation. *J Immunol* 2006; **176**: 7354-7360 [PMID: 16751379 DOI: 10.4049/jimmunol.176.12.7354]

76   **Pan L**, Zhang L, Zhang W, Wu X, Li Y, Yan B, Zhu X, Liu X, Yang C, Xu J, Zhou G, Xu A, Li H, Liu Y. A genome-wide association study identifies polymorphisms in the HLA-DR region associated with non-response to hepatitis B vaccination in Chinese Han populations. *Hum Mol Genet* 2014; **23**: 2210-2219 [PMID: 24282030 DOI: 10.1093/hmg/ddt586]

77   **Li Y**, Ni R, Song W, Shao W, Shrestha S, Ahmad S, Cunningham CK, Flynn PM, Kapogiannis BG, Wilson CM, Tang J. Clear and independent associations of several HLA-DRB1 alleles with differential antibody responses to hepatitis B vaccination in youth. *Hum Genet* 2009; **126**: 685-696 [PMID: 19597844 DOI: 10.1007/s00439-009-0720-z]

78   **Li ZK**, Nie JJ, Li J, Zhuang H. The effect of HLA on immunological response to hepatitis B vaccine in healthy people: a meta-analysis. *Vaccine* 2013; **31**: 4355-4361 [PMID: 23887040 DOI: 10.1016/j.vaccine.2013.06.108]

79   **Yan ZH**, Fan Y, Wang XH, Mao Q, Deng GH, Wang YM. Relationship between HLA-DR gene polymorphisms and outcomes of hepatitis B viral infections: a meta-analysis. *World J Gastroenterol* 2012; **18**: 3119-3128 [PMID: 22791948 DOI: 10.3748/wjg.v18.i24.3119]

80   **Doganay L**, Fejzullahu A, Katrinli S, Yilmaz Enc F, Ozturk O, Colak Y, Ulasoglu C, Tuncer I, Dinler Doganay G. Association of human leukocyte antigen DQB1 and DRB1 alleles with chronic hepatitis B.

World J Gastroenterol 2014; **20**: 8179-8186 [PMID: 25009391 DOI: 10.3748/wjg.v20.i25.8179]

81   **Wu TW**, Chen CF, Lai SK, Lin HH, Chu CC, Wang LY. SNP rs7770370 in HLA-DPB1 loci as a major genetic determinant of response to booster hepatitis B vaccination: results of a genome-wide association study. *J Gastroenterol Hepatol* 2015; **30**: 891-899 [PMID: 25389088 DOI: 10.1111/jgh.12845]

82   **Sakai A**, Noguchi E, Fukushima T, Tagawa M, Iwabuchi A, Kita M, Kakisaka K, Miyasaka A, Takikawa Y, Sumazaki R. Identification of amino acids in antigen-binding site of class Ⅱ HLA proteins independently associated with hepatitis B vaccine response. *Vaccine* 2017; **35**: 703-710 [PMID: 28043736 DOI: 10.1016/j.vaccine.2016.08.068]

83   **Roh EY**, Yoon JH, In JW, Lee N, Shin S, Song EY. Association of HLA-DP variants with the responsiveness to Hepatitis B virus vaccination in Korean Infants. *Vaccine* 2016; **34**: 2602-2607 [PMID: 27083422 DOI: 10.1016/j.vaccine.2016.03.090]

84   **Okada Y**, Uno N, Sato S, Mori S, Sasaki D, Kaku N, Kosai K, Morinaga Y, Hasegawa H, Yanagihara K. Strong influence of human leukocyte antigen-DP variants on response to hepatitis B vaccine in a Japanese population. *Vaccine* 2017; **35**: 5662-5665 [PMID: 28882445 DOI: 10.1016/j.vaccine.2017.08.045]

85   **Al-Qahtani A**, Khalak HG, Alkuraya FS, Al-hamoudi W, Alswat K, Al Balwi MA, Al Abdulkareem I, Sanai FM, Abdo AA. Genome-wide association study of chronic hepatitis B virus infection reveals a novel candidate risk allele on 11q22.3. *J Med Genet* 2013; **50**: 725-732 [PMID: 24065354 DOI: 10.1136/jmedgenet-2013-101724]

86   **Tan W**, Xia J, Dan Y, Li M, Lin S, Pan X, Wang H, Tang Y, Liu N, Tan S, Liu M, He W, Zhang W, Mao Q, Wang Y, Deng G. Genome-wide association study identifies HLA-DR variants conferring risk of HBV-related acute-on-chronic liver failure. *Gut* 2018; **67**: 757-766 [PMID: 28130311 DOI: 10.1136/gutjnl-2016-313035]

87   **Han Y**, Jiang ZY, Jiao LX, Yao C, Lin QF, Ma N, Ju RQ, Yang F, Yu JH, Chen L. Association of human leukocyte antigen-DRB1 alleles with chronic hepatitis B virus infection in the Han Chinese of Northeast China. *Mol Med Rep* 2012; **5**: 1347-1351 [PMID: 22367481 DOI: 10.3892/mmr.2012.800]

88   **Zhang H**, Zhai Y, Hu Z, Wu C, Qian J, Jia W, Ma F, Huang W, Yu L, Yue W, Wang Z, Li P, Zhang Y, Liang R, Wei Z, Cui Y, Xie W, Cai M, Yu X, Yuan Y, Xia X, Zhang X, Yang H, Qiu W, Yang J, Gong F, Chen M, Shen H, Lin D, Zeng YX, He F, Zhou G. Genome-wide association study identifies 1p36.22 as a new susceptibility locus for hepatocellular carcinoma in chronic hepatitis B virus carriers. *Nat Genet* 2010; **42**: 755-758 [PMID: 20676096 DOI: 10.1038/ng.638]

89   **Li S**, Qian J, Yang Y, Zhao W, Dai J, Bei JX, Foo JN, McLaren PJ, Li Z, Yang J, Shen F, Liu L, Yang J, Li S, Pan S, Wang Y, Li W, Zhai X, Zhou B, Shi L, Chen X, Chu M, Yan Y, Wang J, Cheng S, Shen J, Jia W, Liu J, Yang J, Wen Z, Li A, Zhang Y, Zhang G, Luo X, Qin H, Chen M, Wang H, Jin L, Lin D, Shen H, He L, de Bakker PI, Wang H, Zeng YX, Wu M, Hu Z, Shi Y, Liu J, Zhou W. GWAS identifies novel susceptibility loci on 6p21.32 and 21q21.3 for hepatocellular carcinoma in chronic hepatitis B virus carriers. *PLoS Genet* 2012; **8**: e1002791 [PMID: 22807686 DOI: 10.1371/journal.pgen.1002791]

90   **Jiang DK**, Sun J, Cao G, Liu Y, Lin D, Gao YZ, Ren WH, Long XD, Zhang H, Ma XP, Wang Z, Jiang W, Chen TY, Gao Y, Sun LD, Long JR, Huang HX, Wang D, Yu H, Zhang P, Tang LS, Peng B, Cai H, Liu TT, Zhou P, Liu F, Lin X, Tao S, Wan B, Sai-Yin HX, Qin LX, Yin J, Liu L, Wu C, Pei Y, Zhou YF, Zhai Y, Lu PX, Tan A, Zuo XB, Fan J, Chang J, Gu X, Wang NJ, Li Y, Liu YK, Zhai K, Zhang H, Hu Z, Liu J, Yi Q, Xiang Y, Shi R, Ding Q, Zheng W, Shu XO, Mo Z, Shugart YY, Zhang XJ, Zhou G, Shen H, Zheng SL, Xu J, Yu L. Genetic variants in STAT4 and HLA-DQ genes confer risk of hepatitis B virus-related hepatocellular carcinoma. *Nat Genet* 2013; **45**: 72-75 [PMID: 23242368 DOI: 10.1038/ng.2483]

91   **Chan KY**, Wong CM, Kwan JS, Lee JM, Cheung KW, Yuen MF, Lai CL, Poon RT, Sham PC, Ng IO. Genome-wide association study of hepatocellular carcinoma in Southern Chinese patients with chronic hepatitis B virus infection. *PLoS One* 2011; **6**: e28798 [PMID: 22174901 DOI: 10.1371/journal.pone.0028798]

92   **Qu LS**, Jin F, Guo YM, Liu TT, Xue RY, Huang XW, Xu M, Chen

TY, Ni ZP, Shen XZ. Nine susceptibility loci for hepatitis B virus-related hepatocellular carcinoma identified by a pilot two-stage genome-wide association study. *Oncol Lett* 2016; **11**: 624-632 [PMID: 26870257 DOI: 10.3892/ol.2015.3958]

93 **Jemal A**, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90 [PMID: 21296855 DOI: 10.3322/caac.20107]

94 **Munirajan AK**, Ando K, Mukai A, Takahashi M, Suenaga Y, Ohira M, Koda T, Hirota T, Ozaki T, Nakagawara A. KIF1Bbeta functions as a haploinsufficient tumor suppressor gene mapped to chromosome 1p36.2 by inducing apoptotic cell death. *J Biol Chem* 2008; **283**: 24426-24434 [PMID: 18614535 DOI: 10.1074/jbc.M802316200]

95 **Nguyen KB**, Watford WT, Salomon R, Hofmann SR, Pien GC, Morinobu A, Gadina M, O'Shea JJ, Biron CA. Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection. *Science* 2002; **297**: 2063-2066 [PMID: 12242445 DOI: 10.1126/science.1074900]

96 **Chen K**, Shi W, Xin Z, Wang H, Zhu X, Wu X, Li Z, Li H, Liu Y. Replication of genome wide association studies on hepatocellular carcinoma susceptibility loci in a Chinese population. *PLoS One* 2013; **8**: e77315 [PMID: 24204805 DOI: 10.1371/journal.pone.0077315]

97 **Sawai H**, Nishida N, Mbarek H, Matsuda K, Mawatari Y, Yamaoka M, Hige S, Kang JH, Abe K, Mochida S, Watanabe M, Kurosaki M, Asahina Y, Izumi N, Honda M, Kaneko S, Tanaka E, Matsuura K, Itoh Y, Mita E, Korenaga M, Hino K, Murawaki Y, Hiasa Y, Ide T, Ito K, Sugiyama M, Ahn SH, Han KH, Park JY, Yuen MF, Nakamura Y, Tanaka Y, Mizokami M, Tokunaga K. No association for Chinese HBV-related hepatocellular carcinoma susceptibility SNP in other East Asian populations. *BMC Med Genet* 2012; **13**: 47 [PMID: 22712471 DOI: 10.1186/1471-2350-13-47]

98 **Sopipong W**, Tangkijvanich P, Payungporn S, Posuwan N, Poovorawan Y. The KIF1B (rs17401966) single nucleotide polymorphism is not associated with the development of HBV-related hepatocellular carcinoma in Thai patients. *Asian Pac J Cancer Prev* 2013; **14**: 2865-2869 [PMID: 23803045 DOI: 10.7314/APJCP.2013.14.5.2865]

99 **Zhong R**, Tian Y, Liu L, Qiu Q, Wang Y, Rui R, Yang BF, Duan SY, Shi JX, Miao XP, Wang L, Li H. HBV-related hepatocellular carcinoma susceptibility gene KIF1B is not associated with development of chronic hepatitis B. *PLoS One* 2012; **7**: e28839 [PMID: 22363396 DOI: 10.1371/journal.pone.0028839]

100 **Al-Qahtani A**, Al-Anazi M, Viswan NA, Khalaf N, Abdo AA, Sanai FM, Al-Ashgar H, Al-Ahdal M. Role of single nucleotide polymorphisms of KIF1B gene in HBV-associated viral hepatitis. *PLoS One* 2012; **7**: e45128 [PMID: 23028799 DOI: 10.1371/journal.pone.0045128]

101 **Jiang DK**, Ma XP, Wu X, Peng L, Yin J, Dan Y, Huang HX, Ding DL, Zhang LY, Shi Z, Zhang P, Yu H, Sun J, Lilly Zheng S, Deng G, Xu J, Liu Y, Guo J, Cao G, Yu L. Genetic variations in STAT4,C2,HLA-DRB1 and HLA-DQ associated with risk of hepatitis B virus-related liver cirrhosis. *Sci Rep* 2015; **5**: 16278 [PMID: 26538132 DOI: 10.1038/srep16278]

102 **Clark A**, Gerlach F, Tong Hv, Hoan NX, Song le H, Toan NL, Bock CT, Kremsner PG, Velavan TP. A trivial role of STAT4 variant in chronic hepatitis B induced hepatocellular carcinoma. *Infect Genet Evol* 2013; **18**: 257-261 [PMID: 23748017 DOI: 10.1016/j.meegid.2013.05.025]

103 **Kim LH**, Cheong HS, Namgoong S, Kim JO, Kim JH, Park BL, Cho SW, Park NH, Cheong JY, Koh I, Shin HD, Kim YJ. Replication of genome wide association studies on hepatocellular carcinoma susceptibility loci of STAT4 and HLA-DQ in a Korean population. *Infect Genet Evol* 2015; **33**: 72-76 [PMID: 25913043 DOI: 10.1016/j.meegid.2015.04.013]

104 **Lu Y**, Zhu Y, Peng J, Wang X, Wang F, Sun Z. STAT4 genetic polymorphisms association with spontaneous clearance of hepatitis B virus infection. *Immunol Res* 2015; **62**: 146-152 [PMID: 25829184 DOI: 10.1007/s12026-015-8645-1]

105 **Wen J**, Song C, Jiang D, Jin T, Dai J, Zhu L, An J, Liu Y, Ma S, Qin N, Liang C, Chen J, Jiang Y, Yang L, Liu J, Liu L, Geng T, Chen C, Jiang J, Chen J, Zhu F, Zhu Y, Yu L, Shen H, Zhai X, Xu J, Hu Z. Hepatitis B virus genotype, mutations, human leukocyte antigen polymorphisms and their interactions in hepatocellular carcinoma: a multi-centre case-control study. *Sci Rep* 2015; **5**: 16489 [PMID: 26568165 DOI: 10.1038/srep16489]

106 **Ji X**, Zhang Q, Li B, Du Y, Yin J, Liu W, Zhang H, Cao G. Impacts of human leukocyte antigen DQ genetic polymorphisms and their interactions with hepatitis B virus mutations on the risks of viral persistence, liver cirrhosis, and hepatocellular carcinoma. *Infect Genet Evol* 2014; **28**: 201-209 [PMID: 25281206 DOI: 10.1016/j.meegid.2014.09.032]

107 **Zhang X**, Zheng C, Zhou ZH, Li M, Gao YT, Jin SG, Sun XH, Gao YQ. Relationship between HLA-DP gene polymorphisms and the risk of hepatocellular carcinoma: a meta-analysis. *Genet Mol Res* 2015; **14**: 15553-15563 [PMID: 26634522 DOI: 10.4238/2015.December.1.6]

108 **Hai H**, Tamori A, Kawada N. Role of hepatitis B virus DNA integration in human hepatocarcinogenesis. *World J Gastroenterol* 2014; **20**: 6236-6243 [PMID: 24876744 DOI: 10.3748/wjg.v20.i20.6236]

109 **Katrinli S**, Ozdil K, Sahin A, Ozturk O, Kir G, Baykal AT, Akgun E, Sarac OS, Sokmen M, Doğanay HL, Dinler Doğanay G. Proteomic profiling of HBV infected liver biopsies with different fibrotic stages. *Proteome Sci* 2017; **15**: 7 [PMID: 28439208 DOI: 10.1186/s12953-017-0114-4]

110 **Casper M**, Grünhage F, Lammert F. Cancer risk in chronic hepatitis B: Do genome-wide association studies hit the mark? *Hepatology* 2011; **53**: 1390-1392 [PMID: 21480342 DOI: 10.1002/hep.24241]

111 **Budhu A**, Wang XW. Power play: scoring our goals for liver cancer with better GWAS study design. *J Hepatol* 2011; **54**: 823-824 [PMID: 21167853 DOI: 10.1016/j.jhep.2010.10.035]

112 **Zhu H**, Wu J, Shen X. Genome-wide association study: new genetic insights into HBV/HCV-related hepatocellular carcinoma genomes. *Scand J Gastroenterol* 2017; **52**: 209-215 [PMID: 27797287 DOI: 10.1080/00365521.2016.1245778]

113 **Tai DI**, Jeng WJ, Lin CY. A global perspective on hepatitis B-related single nucleotide polymorphisms and evolution during human migration. *Hepatol Commun* 2017; **1**: 1005-1013 [PMID: 29404438 DOI: 10.1002/hep4.1113]

Current guidelines for the management of non-alcoholic fatty liver disease: A systematic review with comparative analysis

Simona Leoni, Francesco Tovoli, Lucia Napoli, Ilaria Serio, Silvia Ferri, Luigi Bolondi

Simona Leoni, Francesco Tovoli, Lucia Napoli, Ilaria Serio, Silvia Ferri, Luigi Bolondi, Department of Medical and Surgical Sciences (DIMEC), Division of Internal Medicine, University of Bologna, Bologna 40136, Italy

ORCID number: Simona Leoni (0000-0001-8825-9698); Francesco Tovoli (0000-0002-8350-1155); Lucia Napoli (0000-0002-5499-9310); Ilaria Serio (0000-0002-6676-4148); Silvia Ferri (0000-0002-6033-8594); Luigi Bolondi (0000-0002-1021-6388).

Correspondence to: Simona Leoni, MD, PhD, Doctor, Department of Medical and Surgical Sciences (DIMEC), Division of Internal Medicine, University of Bologna, via Massarenti 9, Bologna 40138, Italy. simona.leoni@aosp.bo.it
Telephone: +39-51-2142214
Fax:+39-51-2142725

Abstract

The current epidemic of non-alcoholic fatty liver disease (NAFLD) is reshaping the field of hepatology all around the world. The widespread diffusion of metabolic risk factors such as obesity, type2-diabetes mellitus, and dyslipidemia has led to a worldwide diffusion of NAFLD. In parallel to the increased availability of effective anti-viral agents, NAFLD is rapidly becoming the most common cause of chronic liver disease in Western Countries, and a similar trend is expected in Eastern Countries in the next years. This epidemic and its consequences have prompted experts from all over the word in identifying effective strategies for the diagnosis, management, and treatment of NAFLD. Different scientific societies from Europe, America, and Asia-Pacific regions have proposed guidelines based on the most recent evidence about NAFLD. These guidelines are consistent with the key elements in the management of NAFLD, but still, show significant difference about some critical points. We reviewed the current literature in English language to identify the most recent scientific guidelines about NAFLD with the aim to find and critically analyse the main differences. We distinguished guidelines from 5 different scientific societies whose reputation is worldwide recognised and who are representative of the clinical practice in different geographical regions. Differences were noted in: the definition of NAFLD, the opportunity of NAFLD screening in high-risk patients, the non-invasive test proposed for the diagnosis of NAFLD and the identification of NAFLD patients with advanced fibrosis, in the follow-up protocols and, finally, in the treatment strategy (especially in the proposed pharmacological management). These difference have been discussed in the light of the possible evolution of the scenario of

NAFLD in the next years.

Core tip: Non-alcoholic fatty liver disease (NAFLD) is becoming the most common cause of chronic liver disease. As such, an increasing number of scientific reports are investing this condition. To translate these evidence into clinical practice, international scientific societies have proposed guidelines for the management of NAFLD. In this review, we will critically analyse both the converging and diverging points in the current clinical guidelines of NAFLD, with a particular focus on the diagnostic and therapeutic aspects.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) includes a spectrum of disorders ranging from the simple fatty liver to non-alcoholic steatohepatitis, with increasing fibrosis leading to cirrhosis[1]. The prevalence of NAFLD is alarmingly growing worldwide in adult and children/adolescent populations, with a bidirectional association between NAFLD and metabolic syndrome[2]. Obesity, insulin resistance, type 2 diabetes mellitus, and dyslipidemia are the most relevant metabolic conditions related to this spectrum of diseases[1,2].

Clinicians and researchers from several scientific Associations worldwide put significant efforts into increasing knowledge and developing high-quality International Guidelines to improve the management of NAFLD patients in clinical practice. Multidisciplinary panels of experts in different continents have performed systematic analysis and review of the literature on specified topics in the last years. These efforts have led to the creation and publication of various Guidelines.

This paper aims to review and compare the most recently published International Guidelines for the diagnosis and the management of NAFLD in adult populations, to critically evaluate similarities and discrepancies. In particular, we tried to analyse some critical questions and challenges for clinicians in real life.

LITERATURE SEARCH

We performed a database search on PubMed selecting papers published between January 2016 and January 2018 in the English language. The following keywords and terms were considered: (1) Fatty liver disease (("fatty liver"[MeSH Terms] OR ("fatty"[All Fields] AND "liver"[All Fields]) OR "fatty liver"[All Fields]) AND ("disease"[MeSH Terms] OR "disease"[All Fields])) AND guideline ("guideline"[Publication Type] OR "guidelines as topic"[MeSH Terms] OR "guideline"[All Fields]) *AND management* ("organization and administration" [MeSH Terms] OR ("organization"[All Fields] AND "administration"[All Fields]) OR "organization and administration"[All Fields] OR "management"[All Fields] OR "disease management"[MeSH Terms] OR ("disease" [All Fields] AND "management"[All Fields]) OR "disease management"[All Fields]); (2) Fatty liver disease AND recommendation (("fatty liver"[MeSH Terms] OR ("fatty" [All Fields] AND "liver"[All Fields]) OR "fatty liver"[All Fields]) AND ("disease"[MeSH Terms] OR "disease"[All Fields])) AND recommendation[All Fields]; (3) Fatty liver disease and position paper (("fatty liver"[MeSH Terms] OR ("fatty"[All Fields] AND "liver"[All Fields]) OR "fatty liver"[All Fields]) AND ("disease"[MeSH Terms] OR "disease"[All Fields])) AND (position[All Fields] AND ("paper"[MeSH Terms] OR "paper"[All Fields])).

According to this criteria, 119 papers were identified. As a second step, we excluded papers which were not pertinent to any of the following criteria: (1) Clinical Guidelines related to diagnosis and management of NAFLD in the adult population; (2) clinical Guidelines published by Governmental agencies and Scientific Associations.

According to the selection criteria, out of 119 results of PubMed research, 5 Guidelines were finally included in this analysis. These guidelines are strictly focused on the topic of diagnosis and management of NAFLD in adult, excluding pediatric populations and special groups. In detail, the five selected papers included (from the oldest to the newest date of publication): (1) "EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease" by the European Association for the Study of The Liver (EASL), published in 2016[3]; (2) "Nonalcoholic fatty liver disease (NAFLD): Assessment and management" by the National Institute for Health and Care Excellence (NICE), published in 2016[4]; (3)"Asia-Pacific Working Party on Non-Alcoholic Fatty Liver Disease guidelines" published in 2017[5,6]; (4) Italian Association for the Study of the Liver (AISF). AISF position paper on nonalcoholic fatty liver disease (NAFLD): Updates and future directions, published in 2017[7]; (5) "The diagnosis and Management of Nonalcoholic Fatty Liver Disease: Practice Guidance From the American Association for the Study of Liver Diseases" published in 2018[8].

OPEN QUESTIONS

Definition, classification, and diagnostic criteria of NAFLD
Definition and classification: A definition of NAFLD

Table 1 Diagnostic criteria for non-alcoholic fatty liver disease according to the various guidelines

	EASL	NICE	Asia-Pacific	AISF	AASLD
Required criteria	Steatosis in > 5% of hepatocytes by either imaging or histology No other causes of steatosis Insulin resistance	Excessive fat in the liver No other causes of steatosis No significant alcohol consumption	Hepatic steatosis by either imaging or histology No other causes of steatosis No significant alcohol consumption	Hepatic steatosis on either imaging or histology No other causes of steatosis No significant alcohol consumption	Evidence of hepatic steatosis either by imaging or histology No other causes of steatosis No significant alcohol consumption No coexisting chronic liver disease
Alcohol consumption threshold (men)	30 g/d	30 g/d	2 standard drink/d 140 g/wk	30 g/d	21 standard drink/wk 294 g/wk
Alcohol consumption threshold (women)	20 g/d	20 g/d	1 standard drink/d 70 g/wk	20 g/d	14 standard drink/wk 196 g/wk

EASL: European Association for the Study of the Liver; NICE: National Institute for Health and Care Excellence; AISF: Italian Association for the study of the Liver; AASLD: American Association for the Study of Liver Diseases; MRI: Magnetic resonance imaging.

Figure 1 Main difference between non-alcoholic fatty liver and non-alcoholic steatohepatitis. A: Non-alcoholic fatty liver; B: Non-alcoholic steatohepatitis. NAFL is characterized by minimal inflammatory infiltrate without hepatocyte ballooning (arrow). Instead, NASH is associated with lobular inflammatory infiltrate and hepatocyte degeneration (arrow). NAFL: Non-alcoholic fatty liver; NASH: Non-alcoholic steatohepatitis.

is reported in all Guidelines (Table 1). The characteristic points of NAFLD definition include (1) the evidence of excessive hepatic fat accumulation in the liver parenchyma (detected by imaging techniques or histology); (2) the absence of other secondary causes of hepatic fat. Out of them, to strictly define NAFLD patients a significant ongoing or recent alcohol consumption have to be excluded in all recommendation[3-8].

All recommendations identify some different clinical-pathological entities, according to the progression of hepatic histological changes. Simple steatosis and non-alcoholic steatohepatitis (NASH) are defined in all guidelines[3-8]. In detail simple steatosis, also called non-alcoholic fatty liver (NAFL) includes all of the case characterized by steatosis with minimal or absent lobular inflammation. On the contrary, NASH is characterized by hepatocyte ballooning degeneration, diffused lobular inflammation and fibrosis (Figure 1)

Additionally, EASL Asia-Pacific Guidelines and AISF position paper also underline the problem of NAFLD-related HCC, potentially occurring in patients with NAFLD but without cirrhosis[9,10].

Diagnostic criteria: The role of alcohol: The agreement between the different guidelines is not complete when defining the threshold dose of alcohol consumption. As shown in Table 1, EASL[3], NICE, and AISF guidelines[3,4,7] consider as significant an alcohol consumption > 30 g/d in men and > 20 g/d in women. The AASLD guidance[8] indicate the reasonable threshold for significant alcohol consumption > 21 standard drink on average per week in men and > 14 in women. For Asia-Pacific Guidelines[5] a significant alcohol intake was considered > 7 standard alcoholic drinks/week (70 g ethanol) in women and > 14 (140 g) in men.

Who should be screened for NAFLD?

According to the screening programs adopted for other diseases, systematic screening has to be performed for significant health problem with available diagnostic facilities and accepted treatment. Also, there should be recognisable latent or early symptomatic stage, identifiable with sensitive tests. To adequately perform a screening program, the natural history of the disease should be understood, and the economic burden should

	EASL	NICE	Asia-Pacific	AISF	AASLD
Table 2 Comparative analysis of the recommendations regarding the screening for non-alcoholic fatty liver disease					
Systematic screening	No	No	No	No	No
Screening in high-risk groups	Yes Obesity Metabolic syndrome Abnormal liver enzymes	Yes Obesity Type Ⅱ Diabetes	Yes Obesity Type Ⅱ Diabetes	Not mentioned	No[1]
Screening modality	Yes liver enzymes	No liver enzymes Yes ultrasonography	No liver enzymes Yes ultrasonography Yes transient elastography		

[1]"Active surveillance" (but not screening) suggested for patients with type Ⅱ diabetes mellitus. EASL: European Association for the Study of the Liver; NICE: National Institute for Health and Care Excellence; AISF: Italian Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases.

be suitable.

The international guidelines partially diverge about this topic. This disagreement derives from essential considerations regarding natural history, special groups, diagnosis, and therapy: (1) NAFLD in a common cause of chronic liver disease in general population but cause severe liver disease in a small proportion of affected people[1]; (2)Type Ⅱ diabetes patients have higher prevalence of NAFLD, NASH and advanced fibrosis[11-13]; (3) There is a current lack of effective drug treatment; (4) Liver biopsy is a procedure with related risks; (6) Few cost-effective analysis are available[14].

All these considerations imply a different approach to screening in NAFLD by the Scientific Societies. Only EASL, NICE Asia-Pacific Guidelines[3-5] recommend screening respectively in particular, "high-risk" groups (Table 2). On the contrary, AASLD guidelines emphasise that, to date, there is no evidence of cost-effectiveness to support a NAFLD screening in adults even if they have several metabolic risk factors, instead suggesting a concept of "vigilance" in these populations[8].

Which noninvasive test(s) should be used to diagnose NAFLD?

Worldwide guidelines agree that, whenever NAFLD is suspected, the initial diagnostic workup should include a noninvasive imaging examination to confirm the presence of steatosis and general liver biochemistry[3-8]. Non-invasive assessment should aim first of all to identify NAFLD among patients with metabolic risk factors, and then to monitor disease progression and treatment response, identifying patients with the worst prognosis[3].

Imaging: There is a consensus for using abdominal ultrasound (US) as the first-line examination to identify liver steatosis in patients with increased liver blood exams or suspected NAFLD, in daily clinical practice (Figure 2). The main advantages of US derive from its broad availability and low cost. However, its sensitivity among morbidly obese patients (BMI > 40 kg/m^2) is low, and it may miss the diagnosis when the liver hepatic fat content is < 20%[15,16]. Despite these limitations, EASL and AISF underline how ultrasound can significantly assess moderate and severe steatosis, even if an observ-

er dependency remains[15]. NICE guidelines propose to use liver ultrasound to detect hepatic steatosis for children with metabolic syndrome and type 2 diabetes and to retest it every three years if the first examination is negative[4].

On the other hand, magnetic resonance imaging (MRI), either by proton density fat fraction (^{1}H-MRS) or spectroscopy, remains the gold standard to assess and quantify hepatic steatosis, detecting the amount of liver fat as low as 5%-10%, its use in the clinical practice is still limited. In fact, despite its robust accuracy, its limited availability, high costs and a long time of execution, make the procedure not recommended in the daily clinical setting[17]. Asia-Pacific guidelines specify that ^{1}H-MRS is the best option to quantify even moderate changes in liver fat content in clinical trials, considering its high sensitivity compared to histological-proven liver fat reversal. Similarly, EASL guidelines highlight its role, primarily as screening imaging examination for clinical trials and experimental studies[3].

Another imaging technique used to quantify liver fat content is the ultrasonography-based transient elastography (TE) using continuous attenuation parameter (CAP). This promising tool has shown a good sensitivity, measuring simultaneously liver stiffness, potentially evaluating NAFLD severity at the same setting[13]. However, despite its low cost and rapidity of execution, its role in the clinical practice has still to be defined. In fact, EASL guidelines specify that TE has never been compared with hepatic steatosis measured by ^{1}H-MRS and there are limited data about its ability to discriminate different histological patterns[3]. On the other hand, Asia-Pacific guidelines propose CAP as a useful screening tool for NAFLD diagnosis, as well as for demonstrating improvement in hepatic steatosis after lifestyle intervention and body weight reduction[5].

Conventional liver biochemistry: Although NAFLD may present by standard laboratory liver tests, frequently a slight increase of aspartate aminotransferase (AST) or alanine aminotransferase (ALT) or gamma-glutamyl transpeptidase (GammaGT) is observed. However, all the guidelines agree that normal levels of liver enzymes may not exclude NAFLD, being a not sensitive screening
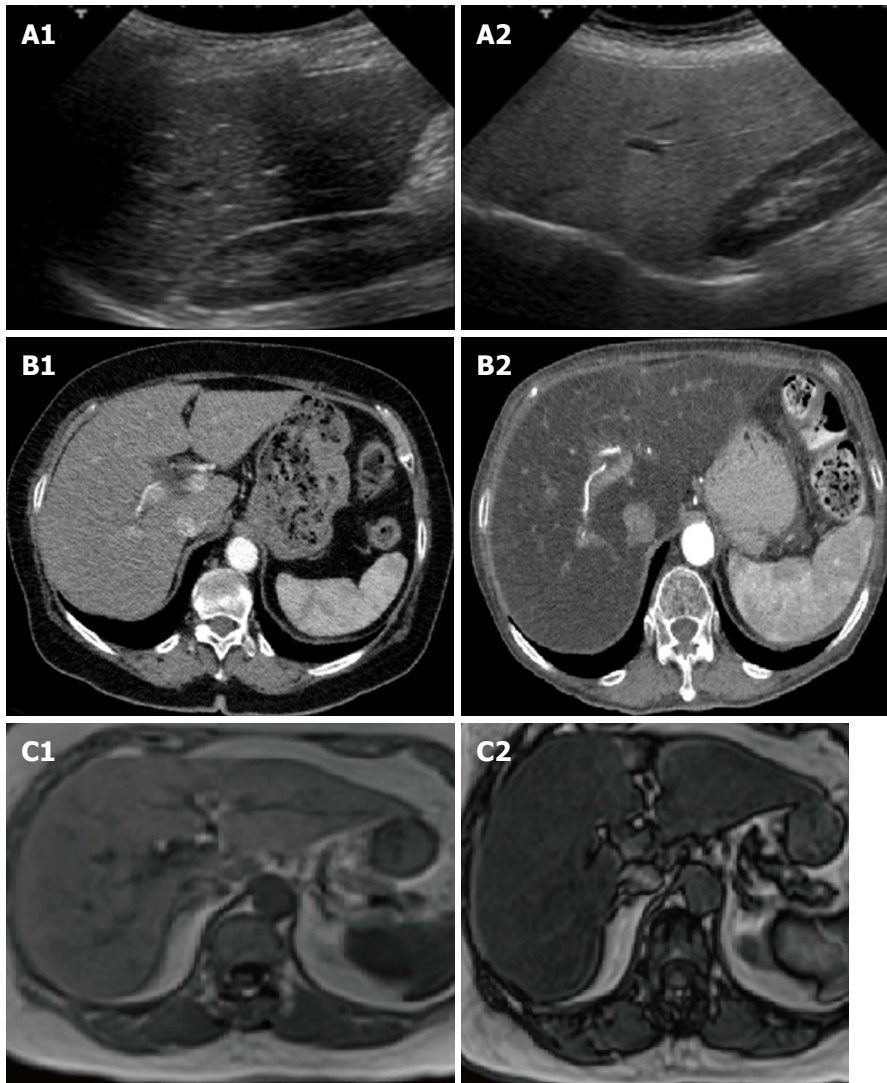
Figure 2 Aspects of liver steatosis according to the different imaging techniques. In normal ultrasound examination liver parenchyma is isoechoic to the renal parenchyma in normal conditions (A1), becoming hyperechoic in presence of liver steatosis (A2). In comparison to a normal liver (B1), a fatty liver appears hypodense compared to the spleen and to the hepatic veins (B2) in computed tomography scans. Finally, in the setting of a severe steatosis, the magnetic resonance signal has a clear fall from in phase (C1) to out phase sequencings (C2).

test[3-8].

Moreover, laboratory alterations may hide another cause of liver disease, in which steatosis is a coexisting condition. On the other hand, detection of abnormalities of laboratory exams (such as ferritin or autoantibodies) not always reflects the presence of another liver disease, but could be an epiphenomenon of NAFLD with no further clinical significance.

In particular, AASLD guidelines underline that elevate serum ferritin and low titers of autoimmune antibodies (especially antinuclear and anti-smooth muscle antibodies) are common features among NAFLD patients[18,19], and may not automatically indicate the presence of hemochromatosis or autoimmune liver disease[8].

Which is the role of diagnostic and prognostic scores?
Noninvasive predictor biomarkers and scores of steatosis and steatohepatitis: The current absence of a highly specific and sensitive noninvasive marker

predicting inflammation and fibrosis is leading to a considerable interest in the identification of new markers of disease progression and to the development of clinical scores of disease severity.

To assess the presence of steatosis, EASL, Asia-Pacific, and Italian guidelines mention the Fatty Liver Index (FLI)[20] and the NAFLD liver fat score[21]. Both of these scores are easily calculated using common blood exams and simple clinical information. In detail, FLI is calculated from serum triglyceride, body mass index, waist circumference, and gamma-glutamyltransferase[20], while NAFLD liver fat score is calculated evaluating the presence/absence of metabolic syndrome and type 2 diabetes, fasting serum insulin, and aminotransferases[21]. They have been validated in a cohort of severely obese patients and the general population, reliably predicting the presence of steatosis, but not its severity[22]. On the contrary, the AASLD guidelines underline that only inflammation and fibrosis dictate the prognosis of NAFLD patients and, consequently, highlight the lack of evidence

of the usefulness of quantifying hepatic steatosis in the routine clinical setting. Instead, AASLD guidelines underline that the simultaneous presence of several metabolic diseases is the most potent predictor of hepatic inflammation and adverse outcome in patients with NAFLD.

The cytokeratin-18 fragment is currently the most studied biomarker to assess the presence of inflammation. Its circulating levels have been largely investigated as a signal of hepatocellular apoptotic activity and therefore as a characteristic feature of NASH[23]. Its role is addressed both by Asia-Pacific and EASL guidelines, which agree that the current evidence does not support its use in clinical practice and that more studies are needed[3,5]. In particular, Asia-Pacific guidelines highlight how increased levels of cytokeratin-18 have good predictive value for NASH *vs* normal livers but do not differentiate NASH *vs* simple steatosis[24,25]. On the other hand, EASL guidelines specify that has been demonstrated that cytokeratin-18 serum levels decrease parallel with histological improvement, but its predictive value is not better than ALT in identifying histological responders[26].

To conclude, guidelines agree that noninvasive tests for detecting NASH and distinguishing it from simple steatosis are not currently available and that liver biopsy remains necessary to detect hepatocyte ballooning and lobular inflammation[3-8].

Noninvasive assessment of advanced fibrosis: Liver fibrosis is considered the leading prognostic factor among patients with NAFLD because of its strong correlation with survival rate and liver-related outcomes[27]. Therefore, NAFLD patients with advanced fibrosis need a closer monitoring and a rigorous adherence to treatment. However, to date, no methods easily performed in daily clinical practice and with a high predictive value for differentiating grades of liver fibrosis have been identified.

Different tools have been investigated at this purpose, including noninvasive scores (NAFLD fibrosis score, Fibrosis 4 calculator, AST/ALT ratio index), serum biomarkers (ELF panel, Fibrometer, Fibrotest, Hepascore) and imaging techniques, such as transient elastography, magnetic resonance elastography (MRE) and shear wave elastography[28].

According to the NICE guideline, the enhanced liver fibrosis (ELF) blood test has shown the best cost-effectiveness in identifying patients with advanced fibrosis stages[29] and therefore should be offered to all patients with an incidental diagnosis of NAFLD[4]. On the other hand, EASL and Italian guidelines suggest the use of NAFLD fibrosis score (NFS) and Fibrosis 4 calculator (FIB-4) as noninvasive scores to identify patients with different risk of advanced fibrosis[3,7]. These two scores have been validated in various ethnically NAFLD patients, predicting liver and cardiovascular-related mortality[30]. Moreover AASLD guidelines highlight that in a recent study both NFS and FIB-4 have shown the best predictive value for advanced fibrosis among

histological proven NAFLD patients in comparison with other scores[28]. EASL guidelines underline that NFS has a stronger negative predictive value for advanced fibrosis than the corresponding positive predictive value[30]. Hence, it should be used for excluding the presence of advanced fibrosis better than stratifying NAFLD patients on different fibrosis stages[3].

Transient elastography has been recently approved by US Food and Drug Administration to investigate adult and pediatric patients with liver disease. Its cut-off value for advanced fibrosis for adults with NAFLD has been established to 9.9 KpA with 95% sensitivity and 77% specificity[31]. In particular, elastography score has been shown to have good diagnostic accuracy for the presence of clinically significant fibrosis, with an AUROC of 0.93 (95%CI: 0.89-0.096) for advanced fibrosis ($\geq$ F3) and cirrhosis, and with a negative predictive value of 90% in ruling out cirrhosis when using a cut-off of 7.9 kPa. However, the ability in differentiating between F2 and F3 fibrosis seems less robust. Because of this high rate of false-positive results, EASL and Asia-Pacific guidelines point out that its low specificity limits its use in daily practice in diagnosing advanced grade of fibrosis and cirrhosis, as well as by a high failure rate[5]. Moreover considering the unreliable results among patients with high BMI and thoracic fold thickness, EASL guidelines highlight that it should not be used alone as first-line detection tool to identify advanced fibrosis or cirrhosis[3]. In this setting, its poor performance can be improved by using M or XL-probe, increasing the success rate[32,33].

American guidelines underline the vital role of magnetic resonance elastography (MRE) in identifying different degrees of fibrosis in patients with NAFLD, performing better than transient elastography for recognising intermediate stage of fibrosis, but showing a same predictive value for advanced fibrosis stages[34]. Therefore AASLD guidelines conclude that MRE and transient elastography are both useful tools for identifying NAFLD patients with advanced liver fibrosis.

On the other hand, shear wave elastography, in the same way as transient elastography, seems to be inappropriate to discriminate between intermediate stages of fibrosis and provide reliable results only in 73% of patients with BMI $\geq$ 30 kg/m^2[35].

Which are the best diagnostic algorithms and follow-up strategies?

The optimal strategy for stratifying NAFLD patients and follow disease progression has not been yet established. According to EASL and Italian guidelines, the combination of noninvasive scores (NFS and FIB-4) and transient elastography should be used to identify patients at low risk of advanced liver disease and for clinical decision-making. Moreover, this combination may instead identify patients who should undergo a liver biopsy to confirm advanced fibrosis, and in whom a more intensive approach is needed)[3,7]. Noninvasive serum scores should be calculated for every patient with

NAFLD to exclude the presence of significant fibrosis. If it cannot be ruled out, then transient elastography should be performed. Hence, if advanced fibrosis is suspected, liver biopsy should be performed for final diagnosis[3,7]. Moreover, a clinical, laboratory and instrumental follow-up for noninvasive monitoring of fibrosis, is suggested every two years for NAFLD patients with normal liver enzymes and low risk of advanced fibrosis. Patients with evidence of NASH or fibrosis should be screened annually and those with cirrhosis every six months, to perform HCC surveillance[3,7].

Similarly, the AASLD guidelines consider NFS, FIB-4, transient elastography, and MRE as the first-line examination to detect patients with advanced fibrosis[8]. Differently from the EASL guidance, however, no diagnostic algorithms or follow up strategies are provided.

The Asia-Pacific guidelines also agree that combined use of serum tests and imaging tools may offer more reliable information than using either method alone[6]. However, they do not specify which noninvasive test is best.

According to the NICE guidelines, every patient with an incidental finding of NAFLD should be screened for advanced fibrosis by ELF blood test. If negative, it should be repeated every three years for adults and two years for children. Moreover, children and young people with type 2 diabetes mellitus or metabolic syndrome, but without steatosis at ultrasound examination, should be reevaluated every three years[4].

Who should undergo liver biopsy?

To date, liver biopsy is the gold standard for diagnosing NASH and staging liver fibrosis, despite several limitations such as sampling error, variability in interpretation by pathologists, high cost and patient discomfort[36]. The "NAFLD Activity Score" (NAS)[37] and the "Steatosis Activity Fibrosis" (SAF) scoring system[38] are recommended to assess disease activity[8].

Except for the NICE guidelines (which do not provide specific indications about which patients should undergo liver biopsy), all of the remaining guidelines substantially agree that confirmatory liver biopsy should not be performed in every NAFLD patients. Instead, it should be reserved for the following two situations: (1) Uncertain diagnosis; (2) suspect of NAFLD-related advanced liver disease.

The AASLD guidelines suggest to perform liver biopsy in patients with metabolic syndrome who are at increased risk of liver inflammation, or when NFS, FIB-4 or liver stiffness measured by transient elastography or MRE suggest the presence of advanced liver fibrosis. In that case, patients would benefit the most from diagnosis, obtaining crucial prognostic information[8].

Similarly, EASL and Italian guidelines recommend performing a liver biopsy when both serum and imaging noninvasive tools show a medium/high risk of advanced liver disease, with the aim to confirm the presence of advanced liver fibrosis. Furthermore, they underline that in selected NAFLD patients at high risk of disease progression, the repetition of liver biopsy should be considered case-by-case every five years[3,7]. On the other hand, the Asia-Pacific guidelines recommend biopsy only when a competing aetiology of chronic liver disease cannot be excluded just by laboratory exams and personal anamnesis, or results of noninvasive tests are inconclusive[5,6].

How to treat NAFLD?

Lifestyle changes: Lifestyle modification consisting of diet, exercise, and weight loss has been advocated to treat patients with NAFLD in all guidelines (Tables 3 and 4). Indeed, weight loss has been reported as a keystone element in improving the histology features of NASH[39,40].

According to the AISF position paper[7], the best therapeutic approach is an adequate lifestyle change focused on weight loss and achieved by physical activity (aerobic activities and resistance training) and healthy diet. In particular, an energy restriction obtained with a low calorie (1200-1600 kcal/d), low fat (less than 10% of saturated fatty acid), low carbohydrate diet (< 50% of total kcal) is suggested. A Mediterranean diet is recommended as the most effective dietary option to induce a weight loss together with beneficial effects on all cardio-metabolic risk factors associated with NAFLD[7].

The Asia-Pacific guidelines agree with a lifestyle intervention strategy for the treatment of NAFLD, focusing the attention on the timing of weight loss that should be gradual because of the deleterious effect of crash diets on NASH. Very low-calorie diets are considered unsustainable, and any specific regimen is preferred over the others[6].

Also, the EASL[3], NICE[4], and AASLD[8] guidelines recommend structured programmes aimed at lifestyle changes towards a healthy diet and habitual physical activity. According to all of these guidelines, a 7%-10% weight loss is the target of most lifestyle interventions.

Pharmacological treatment: (1) Who to treat: According to the EASL guidelines[3], pharmacological therapy should be reserved for: Progressive NASH (bridging fibrosis and cirrhosis); early-stage NASH at high risk for disease progression (age > 50 years, metabolic syndrome, diabetes mellitus or increased ALT)[41]; active NASH with high necroinflammatory activities[42]. Similarly, in the AASLD and Asia-Pacific guidelines, a pharmacological approach is recommended only for patients with NASH and fibrosis[8]. In the NICE guidance, just people with an advanced liver fibrosis (ELF test > 10.51) are proposed for pharmacological treatment[4]. In the AISF position paper, drug therapy is suggested for patients who are at high risk for disease progression[7]. (2) Pharmacologic treatment: Currently, no drugs have been approved for the treatment of NASH by the US Food and Drug Administration or by the European Medicines Agency. All guidelines acknowledge that any medicines

Table 3 Comparison of recommendations about non-invasive evaluation of fibrosis and follow up strategies

	EASL	NICE	Asia-Pacific	AISF	AASLD
Non-invasive evaluation	NFS and FIB-4 upon diagnosis. If inconclusive, perform transient elastography	ELF blood test	Combination of serum tests and imaging tools (no specification about the preferred tests)	NFS + FIB-4 upon diagnosis. If inconclusive, perform transient elastography	NFS, FIB-4 and transient elastography (or MRE) upon diagnosis
Follow up	Negative markers > reassess every 2 yr; Fibrosis or abnormal liver enzymes > reassess every year; Cirrhosis-> surveillance every 6 mo	Negative ELF test, > reassess every 3 yr; Positive ELF test > liver biopsy	No information provided	Negative markers > reassess every 2 yr; Fibrosis or abnormal liver enzymes > reassess every year; Cirrhosis > surveillance every 6 mo	No information provided

EASL: European Association for the Study of the Liver; NICE: National Institute for Health and Care Excellence; AISF: Italian Association for the study of the Liver; AASLD: American Association for the Study of Liver Diseases; NFS: NAFLD fibrosis score; FIB-4: Fibrosis-4; ELF: Enhanced Liver Fibrosis; MRE: Magnetic resonance elastography.

Table 4 Guidance statements about lifestyle interventions

	EASL	NICE	Asia-Pacific	AISF	AASLD
Dietary restrictions	500-1000 kcal deficit; weight loss of 500-1000 g/wk with a 7%-10% total weight loss	Main recommendations on diet of NICE's obesity and preventing excess weight gain guidelines	500-1000 kcal deficit	1200-1600 kcal/d; fat-low (< 30% of total calories); carbohydrate-low (< 50% of total calories)	500-1000 kcal deficit
Physical activity	Aerobic and resistance training (150-200 min/wk in 3-5 sessions)	Main recommendation of on physical activity of NICE's obesity and preventing excess weight gain guidelines	Aerobic and resistance training	Aerobic and resistance training	Aerobic and resistance training (> 150 min/wk)
Gold standard diet	Low-to-moderate fat and moderate-to-high carbohydrate intake Low-carbohydrate ketogenic diets or high-protein Mediterranean diet	No specific suggestions	All, excluding very low-calorie diets	Mediterranean diet	No specific suggestions

EASL: European Association for the Study of the Liver; NICE: National Institute for Health and Care Excellence; AISF: Italian Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases.

prescribed explicitly for NAFLD should be considered as an off-label treatment and that the decision should be discussed with the patient, carefully balancing the benefits and the safety. However, the guidelines are widely discordant about possibly helpful drugs (Table 5).

Metformin: Due to the evidence of its limited efficacy in improving the histological features of NAFLD[43-45], metformin is not recommended by any guidelines to specifically treat NAFLD[3-8].

Pioglitazone: Pioglitazone, a thiazolidinedione, is a peroxisome proliferator-activated receptor (PPAR) gamma agonist with insulin-sensitising effects. Treatment with pioglitazone improves insulin sensitivity, aminotransferases, steatosis, inflammation, and ballooning in patients with NASH and prediabetes or T2DM[46]. The PIVENS trial (a large multicenter RCT) compared low dose pioglitazone (30 mg/d) *vs* vitamin E (800 UI/d) *vs* placebo for two years in patients without overt diabetes. Pioglitazone improved all histological features (except for fibrosis) and achieved resolution of NASH more often than placebo[47]. The histological benefit occurred

together with ALT improvement and partial correction of insulin resistance. The main side effects of glitazones are weight gain[48-51], and bone fractures in women[52]. The use of pioglitazone for the treatment of NAFLD is endorsed both by the NICE and AASLD guidelines, with significant limitations. In the first case, pioglitazone should be prescribed only in second and third level centres, after a careful evaluation[4]. In the latter case, pioglitazone is reserved for patients with biopsy-proven NASH[8]. The EASL guidelines are more cautious, generically suggesting to consider pioglitazone for the treatment of diabetes in patients with a concurrent NAFLD[3]. Even the Asia-Pacific and the Italian guidelines acknowledge the potential benefits of pioglitazone, however, suggest that more evidence should be available before a firm recommendation can be made[6,7].

Vitamin E: Vitamin E is an anti-oxidant and has been investigated to treat NASH. In the PIVENS trial, vitamin E at a dose of 800 IU/d of α-tocopherol for 96 wk was associated with a decrease in serum aminotransferases and histological improvement in steatosis, inflammation,

Table 5 Recommendations about pharmacological treatment of non-alcoholic fatty liver disease

	EASL	NICE	ASIA-PACIFIC	AISF	AASLD
Metformin	Insufficient evidence	Not beneficial	Not beneficial	Not mentioned	Not beneficial
Vitamin E	Insufficient evidence	Consider use regardless of diabetes	Not beneficial	Insufficient evidence	Consider use in non-diabetic, biopsy-proven NASH
PPAR-gamma agonists	Consider use in selected diabetic patients	Consider pioglitazone in adults regardless of diabetes	Insufficient evidence in Asian	Insufficient evidence, potentially useful	Pioglitazone indicated in biopsy-proven NASH (regardless of diabetes)
PUFA	Not beneficial	Insufficient evidence	Not beneficial	Not mentioned	Not beneficial
Pentoxifylline	Insufficient evidence	Not mentioned	Not beneficial	Not mentioned	Not mentioned
GLP-1 analogues	Insufficient evidence, potentially useful	Insufficient evidence	Insufficient evidence in Asian patients	Insufficient evidence, potentially useful	Insufficient evidence
UDCA	Not beneficial	Not beneficial	Not mentioned	Not mentioned	Not beneficial
Obetycolic acid	Scarce evidence	Not mentioned	waiting for ongoing RCT results	Waiting for ongoing RCT results	Insufficient evidence
Silymarin	Not mentioned	Not mentioned	insufficient evidence, potentially useful	Not mentioned	Not mentioned
Statins	Safe but not beneficial	Safe but not beneficial	Safe but not beneficial	Safe but not beneficial	Safe but not beneficial

EASL: European Association for the Study of the Liver; NICE: National Institute for Health and Care Excellence; AISF: Italian Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases; PPAR: Peroxisome proliferator-activated receptors; PUFA: Poly-unsaturated fatty acids; GLP-1: Glucagon-like peptide-1.

and ballooning and resolution of steatohepatitis in adults with NASH[47]. Long-term safety of vitamin E is under dispute, with two different meta-analyses leading to conflicting results when analysing the all-cause mortality in patients treated with t doses of > 800 IU/d[51,52]. Similarly to pioglitazone, vitamin E is recommended by the NICE and AASLD guidelines (limited to biopsy-proven NASH in the latter case)[4,8]. EASL and AISF guidelines call for more evidence before any recommendation[3,7], while Asia-Pacific guidelines advice against the use of vitamin E which is described as not beneficial by the current evidence[6].

Glucagon-like peptide-1 (GLP-1) analogues: Incretin-mimetics, acting on the glucose-insulin interplay have shown favourable results in pre-marketing studies on liver enzymes[53]. Also, in a published randomised, placebo-controlled trial consisting of 52 patients with biopsy-proven NASH, liraglutide administered sub-cutaneously once-daily for 48 wk was associated with greater resolution of NASH and less progression of fibrosis[54]. Both the AASLD and NICE recommendations state that there is still too few evidence to support the use of GLP-1 analogues to specifically treat liver disease in patients with NAFLD[4,8]. The remaining guidelines also agree on this point, however also state that further evidence may prove the efficacy of these drugs. In particular, the APASL guidelines consider some more elements in their recommendations. On the one hand, GLP-1 agonists appeared to reduce glycated haemoglobin more efficiently in Asian patients with type 2 diabetes mellitus[55]. On the other hand, there has been no study on Asian NASH patients, even if the pharmacokinetics of GLP-1 agonists do not appear to differ between Asian and non-Asian patients according to preliminary evidence[56,57].

Statins: Historically, the use of statins in patients with chronic liver diseases has been considered as potentially troublesome due to the risk of hepatotoxicity. At the same time, a considerable portion of NAFLD patients usually receives statins because of their multiple cardiovascular risk factors. Consequently, the primary concern of the guidelines is the safety of statins. In this regard, a recent review underlined the safety of statin and their efficacy in reducing the associated cardiovascular morbidity in patients with NAFLD, including those with slightly elevated alanine transaminases (up to 3 × reference upper limit)[58]. All of the guidelines agree about the safety of prescribing statins (or continuing an ongoing statin therapy) in patients with NAFLD, even with compensated cirrhosis. However, routine prescription of a statin is not recommended in patients with decompensated cirrhosis and acute liver failure[59,60].

Silymarin: Silymarin is a complex mixture of six major flavonolignans (silybins A and B, isosilybins A and B, silychristin, and silydianin), as well as other minor poly-phenolic compounds[61]. In a randomised, double-blinded, placebo-controlled study on patients with biopsy-proven NASH, silymarin dosage of 700 mg three times daily for 48 wk resulted in a significantly higher percentage of fibrosis reduction compared with placebo (22.4% *vs* 6.0%, $P = 0.023$)[62]. The dosage was safe and well tolerated[62]. Silymarin is mentioned as a potentially useful treatment for NASH in Asia-Pacific guidelines only. However, optimal dose and duration still require further studies before a full recommendation[6].

Bariatric surgery: In patients unresponsive to lifestyle changes and pharmacotherapy, bariatric surgery is an option for reducing weight and metabolic complications, with stable results in the long-term[63]. Bariatric surgery can also improve liver histology, both regarding steatosis and ballooning[64,65] and fibrosis[65]. However, the presence

of established cirrhosis is associated with peri-operative risks. In particular, in the analysis performed from the Nationwide Inpatient Sample (1998-2007), mortality was higher in patients with compensated cirrhosis (0.9%) and much higher in those with decompensated cirrhosis (16.3%)[66]. No robust data on the comparative effects of different bariatric procedures on liver fat are available in the literature.

Based on the evidence as mentioned earlier, the EASL guidelines consider bariatric surgery an option in patients unresponsive to lifestyle changes and pharmacotherapy, for reducing weight and metabolic complications[3]. Guidance statements by the AASLD also consider a role of foregut bariatric surgery in otherwise eligible obese individuals with NAFLD or NASH[8].

The Asia-Pacific recommendation limits the role of bariatric surgery only to patients with class II obesity (BMI > 32.5 kg/m^2 in Asians and 35 kg/m^2 in Caucasians)[6]. AISF and NICE guidelines do not mention bariatric surgery.

Liver transplantation: NASH is becoming the most common indication to liver transplantation in Western Countries[67]. Because of the high prevalence of obesity, sarcopenia, cardiovascular disease and chronic kidney disease among patients with NASH, there is a higher frequency of post-transplant complications and increased graft loss[68,69]. Because of the risk of prolonged ventilation, poor wound healing, higher rate of primary graft non-function, and increased infectious complications, patients with severe obesity (BMI > 40 kg/m^2) may even be considered unfit for liver transplantation, unless efforts are made preoperatively to reduce body weight with individualized plans of lifestyle modifications[70].

AISF and NICE guidance do not mention liver transplantation. All of the remaining guidelines agree that liver transplantation is an acceptable procedure in NASH patients with an end-stage liver disease, with the same indications adopted for other etiologies of liver disease[3-8].

CONCLUSION

The comparative analysis of the most recent international guidelines for the management of NAFLD showed some common orientation between the different recommendations, as well as diverging points. The most notable differences involved: the identification of the alcohol threshold defining NAFLD, the screening strategies in high-risk populations, the preferred non-invasive biomarkers for the assessment of advanced fibrosis, and the pharmacological treatment. These differences should not be necessarily seen as a limitation, but rather an expression of the geographical differences in genetic predisposition to NAFLD, lifestyle habits, healthcare systems. Arguably, the similarity in the recommendations could greatly help in ensuring homogenous management of NALFD all over the world, with favourable repercussions both in clinical practice and in clinical trials. In

the next years, we might see a trend toward more homogenous guidelines thanks to the increasing body of evidence. In particular, the advancements in the imaging technologies could lead to new and widely accepted noninvasive methods to assess advanced liver fibrosis. Moreover, some clinical trials are investigating potentially effective drugs. If positive, the currently diverging pharmacological recommendations may reach a higher concordance. NAFLD is becoming a leading field of research in hepatology: new evidence is destined to change the current landscape of knowledge, prompting greater benefits to the patients as well as changes in the recommendations for clinical practice.

REFERENCES

1   **De Minicis S**, Day C, Svegliati-Baroni G. From NAFLD to NASH and HCC: pathogenetic mechanisms and therapeutic insights. *Curr Pharm Des* 2013; **19**: 5239-5249 [PMID: 23394093 DOI: 10.2174/13816128113196290006]

2   **Schwimmer JB**, Deutsch R, Kahen T, Lavine JE, Stanley C, Behling C. Prevalence of fatty liver in children and adolescents. *Pediatrics* 2006; **118**: 1388-1393 [PMID: 17015527 DOI: 10.1542/peds.2006-1212]

3   **European Association for the Study of the Liver (EASL).** European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO). EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *J Hepatol* 2016; **64**: 1388-1402 [PMID: 27062661 DOI: 10.1016/j.jhep.2015.11.004]

4   National Institute for Health and Care Excellence (UK). Non-Alcoholic Fatty Liver Disease: Assessment and Management. Available from: URL: http//www.niceorg.uk\guidance\ng49

5   **Wong VW**, Chan WK, Chitturi S, Chawla Y, Dan YY, Duseja A, Fan J, Goh KL, Hamaguchi M, Hashimoto E, Kim SU, Lesmana LA, Lin YC, Liu CJ, Ni YH, Sollano J, Wong SK, Wong GL, Chan HL, Farrell G. Asia-Pacific Working Party on Non-alcoholic Fatty Liver Disease guidelines 2017-Part 1: Definition, risk factors and assessment. *J Gastroenterol Hepatol* 2018; **33**: 70-85 [PMID: 28670712 DOI: 10.1111/jgh.13857]

6   **Chitturi S**, Wong VW, Chan WK, Wong GL, Wong SK, Sollano J, Ni YH, Liu CJ, Lin YC, Lesmana LA, Kim SU, Hashimoto E, Hamaguchi M, Goh KL, Fan J, Duseja A, Dan YY, Chawla Y, Farrell G, Chan HL. The Asia-Pacific Working Party on Non-alcoholic Fatty Liver Disease guidelines 2017-Part 2: Management and special groups. *J Gastroenterol Hepatol* 2018; **33**: 86-98 [PMID: 28692197 DOI: 10.1111/jgh.13856]

7   **Italian Association for the Study of the Liver (AISF)**. AISF position paper on nonalcoholic fatty liver disease (NAFLD): Updates and future directions. *Dig Liver Dis* 2017; **49**: 471-483 [PMID: 28215516 DOI: 10.1016/j.dld.2017.01.147]

8   **Chalasani N**, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, Harrison SA, Brunt EM, Sanyal AJ. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. *Hepatology* 2018; **67**: 328-357 [PMID: 28714183 DOI: 10.1002/hep.29367]

9   **Paradis V**, Zalinski S, Chelbi E, Guedj N, Degos F, Vilgrain V, Bedossa P, Belghiti J. Hepatocellular carcinomas in patients with metabolic syndrome often develop without significant liver fibrosis: a pathological analysis. *Hepatology* 2009; **49**: 851-859 [PMID: 19115377 DOI: 10.1002/hep.22734]

10 **Piscaglia F**, Svegliati-Baroni G, Barchetti A, Pecorelli A, Marinelli S, Tiribelli C, Bellentani S; HCC-NAFLD Italian Study Group. Clinical patterns of hepatocellular carcinoma in nonalcoholic fatty liver disease: A multicenter prospective study. *Hepatology* 2016; **63**: 827-838 [PMID: 26599351 DOI: 10.1002/hep.28368]

11 **Portillo-Sanchez P**, Bril F, Maximos M, Lomonaco R, Biernacki D,

Orsak B, Subbarayan S, Webb A, Hecht J, Cusi K. High Prevalence of Nonalcoholic Fatty Liver Disease in Patients With Type 2 Diabetes Mellitus and Normal Plasma Aminotransferase Levels. *J Clin Endocrinol Metab* 2015; **100**: 2231-2238 [PMID: 25885947 DOI: 10.1210/jc.2015-1966]

12   **Koehler EM**, Plompen EP, Schouten JN, Hansen BE, Darwish Murad S, Taimr P, Leebeek FW, Hofman A, Stricker BH, Castera L, Janssen HL. Presence of diabetes mellitus and steatosis is associated with liver stiffness in a general population: The Rotterdam study. *Hepatology* 2016; **63**: 138-147 [PMID: 26171685 DOI: 10.1002/hep.27981]

13   **Kwok R**, Choi KC, Wong GL, Zhang Y, Chan HL, Luk AO, Shu SS, Chan AW, Yeung MW, Chan JC, Kong AP, Wong VW. Screening diabetic patients for non-alcoholic fatty liver disease with controlled attenuation parameter and liver stiffness measurements: a prospective cohort study. *Gut* 2016; **65**: 1359-1368 [PMID: 25873639 DOI: 10.1136/gutjnl-2015-309265]

14   **Klebanoff MJ**, Corey KE, Chhatwal J, Kaplan LM, Chung RT, Hur C. Bariatric surgery for nonalcoholic steatohepatitis: A clinical and cost-effectiveness analysis. *Hepatology* 2017; **65**: 1156-1164 [PMID: 27880977 DOI: 10.1002/hep.28958]

15   **Saadeh S**, Younossi ZM, Remer EM, Gramlich T, Ong JP, Hurley M, Mullen KD, Cooper JN, Sheridan MJ. The utility of radiological imaging in nonalcoholic fatty liver disease. *Gastroenterology* 2002; **123**: 745-750 [PMID: 12198701 DOI: 10.1053/gast.2002.35354]

16   **Ryan CK**, Johnson LA, Germin BI, Marcos A. One hundred consecutive hepatic biopsies in the workup of living donors for right lobe liver transplantation. *Liver Transpl* 2002; **8**: 1114-1122 [PMID: 12474149 DOI: 10.1053/jlts.2002.36740]

17   **Szczepaniak LS**, Nurenberg P, Leonard D, Browning JD, Reingold JS, Grundy S, Hobbs HH, Dobbins RL. Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. *Am J Physiol Endocrinol Metab* 2005; **288**: E462-E468 [PMID: 15339742 DOI: 10.1152/ajpendo.00064.2004]

18   **Valenti L**, Fracanzani AL, Bugianesi E, Dongiovanni P, Galmozzi E, Vanni E, Canavesi E, Lattuada E, Roviaro G, Marchesini G, Fargion S. HFE genotype, parenchymal iron accumulation, and liver fibrosis in patients with nonalcoholic fatty liver disease. *Gastroenterology* 2010; **138**: 905-912 [PMID: 19931264 DOI: 10.1053/j.gastro.2009.11.013]

19   **Vuppalanchi R**, Gould RJ, Wilson LA, Unalp-Arida A, Cummings OW, Chalasani N, Kowdley KV; Nonalcoholic Steatohepatitis Clinical Research Network (NASH CRN). Clinical significance of serum autoantibodies in patients with NAFLD: results from the nonalcoholic steatohepatitis clinical research network. *Hepatol Int* 2012; **6**: 379-385 [PMID: 21557024 DOI: 10.1007/s12072-011-9277-8]

20   **Bedogni G**, Bellentani S, Miglioli L, Masutti F, Passalacqua M, Castiglione A, Tiribelli C. The Fatty Liver Index: a simple and accurate predictor of hepatic steatosis in the general population. *BMC Gastroenterol* 2006; **6**: 33 [PMID: 17081293 DOI: 10.1186/1471-230X-6-33]

21   **Kotronen A**, Peltonen M, Hakkarainen A, Sevastianova K, Bergholm R, Johansson LM, Lundbom N, Rissanen A, Ridderstråle M, Groop L, Orho-Melander M, Yki-Järvinen H. Prediction of non-alcoholic fatty liver disease and liver fat using metabolic and genetic factors. *Gastroenterology* 2009; **137**: 865-872 [PMID: 19524579 DOI: 10.1053/j.gastro.2009.06.005]

22   **Fedchuk L**, Nascimbeni F, Pais R, Charlotte F, Housset C, Ratziu V; LIDO Study Group. Performance and limitations of steatosis biomarkers in patients with nonalcoholic fatty liver disease. *Aliment Pharmacol Ther* 2014; **40**: 1209-1222 [PMID: 25267215 DOI: 10.1111/apt.12963]

23   **Feldstein AE**, Wieckowska A, Lopez AR, Liu YC, Zein NN, McCullough AJ. Cytokeratin-18 fragment levels as noninvasive biomarkers for nonalcoholic steatohepatitis: a multicenter validation study. *Hepatology* 2009; **50**: 1072-1078 [PMID: 19585618 DOI: 10.1002/hep.23050]

24   **Shen J**, Chan HL, Wong GL, Chan AW, Choi PC, Chan HY, Chim AM, Yeung DK, Yu J, Chu WC, Wong VW. Assessment of non-alcoholic fatty liver disease using serum total cell death and apoptosis markers. *Aliment Pharmacol Ther* 2012; **36**: 1057-1066 [PMID: 23066946 DOI: 10.1111/apt.12091]

25   **Chan WK**, Sthaneshwar P, Nik Mustapha NR, Mahadeva S. Limited utility of plasma M30 in discriminating non-alcoholic steatohepatitis from steatosis--a comparison with routine biochemical markers. *PLoS One* 2014; **9**: e105903 [PMID: 25184298 DOI: 10.1371/journal.pone.0105903]

26   **Vuppalanchi R**, Jain AK, Deppe R, Yates K, Comerford M, Masuoka HC, Neuschwander-Tetri BA, Loomba R, Brunt EM, Kleiner DE, Molleston JP, Schwimmer JB, Lavine JE, Tonascia J, Chalasani N. Relationship between changes in serum levels of keratin 18 and changes in liver histology in children and adults with nonalcoholic fatty liver disease. *Clin Gastroenterol Hepatol* 2014; **12**: 2121-2130.e1-2 [PMID: 24846279 DOI: 10.1016/j.cgh.2014.05.010]

27   **Ekstedt M**, Hagström H, Nasr P, Fredrikson M, Stål P, Kechagias S, Hultcrantz R. Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up. *Hepatology* 2015; **61**: 1547-1554 [PMID: 25125077 DOI: 10.1002/hep.27368]

28   **Kaswala DH**, Lai M, Afdhal NH. Fibrosis Assessment in Nonalcoholic Fatty Liver Disease (NAFLD) in 2016. *Dig Dis Sci* 2016; **61**: 1356-1364 [PMID: 27017224 DOI: 10.1007/s10620-016-4079-4]

29   **Fagan KJ**, Pretorius CJ, Horsfall LU, Irvine KM, Wilgen U, Choi K, Fletcher LM, Tate J, Melino M, Nusrat S, Miller GC, Clouston AD, Ballard E, O'Rourke P, Lampe G, Ungerer JP, Powell EE. ELF score ≥9.8 indicates advanced hepatic fibrosis and is influenced by age, steatosis and histological activity. *Liver Int* 2015; **35**: 1673-1681 [PMID: 25495373 DOI: 10.1111/liv.12760]

30   **Guha IN**, Parkes J, Roderick P, Chattopadhyay D, Cross R, Harris S, Kaye P, Burt AD, Ryder SD, Aithal GP, Day CP, Rosenberg WM. Noninvasive markers of fibrosis in nonalcoholic fatty liver disease: Validating the European Liver Fibrosis Panel and exploring simple markers. *Hepatology* 2008; **47**: 455-460 [PMID: 18038452 DOI: 10.1002/hep.21984]

31   **Tapper EB**, Challies T, Nasser I, Afdhal NH, Lai M. The Performance of Vibration Controlled Transient Elastography in a US Cohort of Patients With Nonalcoholic Fatty Liver Disease. *Am J Gastroenterol* 2016; **111**: 677-684 [PMID: 26977758 DOI: 10.1038/ajg.2016.49]

32   **Wong VW**, Vergniol J, Wong GL, Foucher J, Chan AW, Chermak F, Choi PC, Merrouche W, Chu SH, Pesque S, Chan HL, de Lédinghen V. Liver stiffness measurement using XL probe in patients with nonalcoholic fatty liver disease. *Am J Gastroenterol* 2012; **107**: 1862-1871 [PMID: 23032979 DOI: 10.1038/ajg.2012.331]

33   **Wong VW**, Vergniol J, Wong GL, Foucher J, Chan HL, Le Bail B, Choi PC, Kowo M, Chan AW, Merrouche W, Sung JJ, de Lédinghen V. Diagnosis of fibrosis and cirrhosis using liver stiffness measurement in nonalcoholic fatty liver disease. *Hepatology* 2010; **51**: 454-462 [PMID: 20101745 DOI: 10.1002/hep.23312]

34   **Imajo K**, Kessoku T, Honda Y, Tomeno W, Ogawa Y, Mawatari H, Fujita K, Yoneda M, Taguri M, Hyogo H, Sumida Y, Ono M, Eguchi Y, Inoue T, Yamanaka T, Wada K, Saito S, Nakajima A. Magnetic Resonance Imaging More Accurately Classifies Steatosis and Fibrosis in Patients With Nonalcoholic Fatty Liver Disease Than Transient Elastography. *Gastroenterology* 2016; **150**: 626-637.e7 [PMID: 26677985 DOI: 10.1053/j.gastro.2015.11.048]

35   **Cheah MC**, McCullough AJ, Goh GB. Current Modalities of Fibrosis Assessment in Non-alcoholic Fatty Liver Disease. *J Clin Transl Hepatol* 2017; **5**: 261-271 [PMID: 28936407 DOI: 10.14218/JCTH.2017.00009]

36   **Kleiner DE**, Brunt EM. Nonalcoholic fatty liver disease: pathologic patterns and biopsy evaluation in clinical research. *Semin Liver Dis* 2012; **32**: 3-13 [PMID: 22418883 DOI: 10.1055/s-0032-1306421]

37   **Bedossa P**, Poitou C, Veyrie N, Bouillot JL, Basdevant A, Paradis V, Tordjman J, Clement K. Histopathological algorithm and scoring system for evaluation of liver lesions in morbidly obese patients. *Hepatology* 2012; **56**: 1751-1759 [PMID: 22707395 DOI: 10.1002/hep.25889]

38   **Bedossa P**; FLIP Pathology Consortium. Utility and appropriateness of the fatty liver inhibition of progression (FLIP) algorithm and steatosis, activity, and fibrosis (SAF) score in the evaluation of biopsies of nonalcoholic fatty liver disease. *Hepatology* 2014; **60**: 565-575 [PMID: 24753132 DOI: 10.1002/hep.27173]

39   **Haufe S**, Engeli S, Kast P, Böhnke J, Utz W, Haas V, Hermsdorf M, Mähler A, Wiesner S, Birkenfeld AL, Sell H, Otto C, Mehling H, Luft FC, Eckel J, Schulz-Menger J, Boschmann M, Jordan J. Randomized comparison of reduced fat and reduced carbohydrate hypocaloric diets on intrahepatic fat in overweight and obese human subjects. *Hepatology* 2011; **53**: 1504-1514 [PMID: 21400557 DOI: 10.1002/hep.24242]

40   **Asrih M**, Jornayvaz FR. Diets and nonalcoholic fatty liver disease: the good and the bad. *Clin Nutr* 2014; **33**: 186-190 [PMID: 24262589 DOI: 10.1016/j.clnu.2013.11.003]

41   **Adams LA**, Sanderson S, Lindor KD, Angulo P. The histological course of nonalcoholic fatty liver disease: a longitudinal study of 103 patients with sequential liver biopsies. *J Hepatol* 2005; **42**: 132-138 [PMID: 15629518 DOI: 10.1016/j.jhep.2004.09.012]

42   **Sanyal AJ**, Friedman SL, McCullough AJ, Dimick-Santos L; American Association for the Study of Liver Diseases; United States Food and Drug Administration. Challenges and opportunities in drug and biomarker development for nonalcoholic steatohepatitis: findings and recommendations from an American Association for the Study of Liver Diseases-U.S. Food and Drug Administration Joint Workshop. *Hepatology* 2015; **61**: 1392-1405 [PMID: 25557690 DOI: 10.1002/hep.27678]

43   **Bugianesi E**, Gentilcore E, Manini R, Natale S, Vanni E, Villanova N, David E, Rizzetto M, Marchesini G. A randomized controlled trial of metformin versus vitamin E or prescriptive diet in nonalcoholic fatty liver disease. *Am J Gastroenterol* 2005; **100**: 1082-1090 [PMID: 15842582 DOI: 10.1111/j.1572-0241.2005.41583.x]

44   **Haukeland JW**, Konopski Z, Eggesbø HB, von Volkmann HL, Raschpichler G, Bjøro K, Haaland T, Løberg EM, Birkeland K. Metformin in patients with non-alcoholic fatty liver disease: a randomized, controlled trial. *Scand J Gastroenterol* 2009; **44**: 853-860 [PMID: 19811343 DOI: 10.1080/00365520902845268]

45   **Shields WW**, Thompson KE, Grice GA, Harrison SA, Coyle WJ. The Effect of Metformin and Standard Therapy versus Standard Therapy alone in Nondiabetic Patients with Insulin Resistance and Nonalcoholic Steatohepatitis (NASH): A Pilot Trial. *Therap Adv Gastroenterol* 2009; **2**: 157-163 [PMID: 21180541 DOI: 10.1177/1756283X09105462]

46   **Belfort R**, Harrison SA, Brown K, Darland C, Finch J, Hardies J, Balas B, Gastaldelli A, Tio F, Pulcini J, Berria R, Ma JZ, Dwivedi S, Havranek R, Fincke C, DeFronzo R, Bannayan GA, Schenker S, Cusi K. A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. *N Engl J Med* 2006; **355**: 2297-2307 [PMID: 17135584 DOI: 10.1056/NEJMoa060326]

47   **Sanyal AJ**, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, Neuschwander-Tetri BA, Lavine JE, Tonascia J, Unalp A, Van Natta M, Clark J, Brunt EM, Kleiner DE, Hoofnagle JH, Robuck PR; NASH CRN. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med* 2010; **362**: 1675-1685 [PMID: 20427778 DOI: 10.1056/NEJMoa0907929]

48   **Cusi K**, Orsak B, Bril F, Lomonaco R, Hecht J, Ortiz-Lopez C, Tio F, Hardies J, Darland C, Musi N, Webb A, Portillo-Sanchez P. Long-Term Pioglitazone Treatment for Patients With Nonalcoholic Steatohepatitis and Prediabetes or Type 2 Diabetes Mellitus: A Randomized Trial. *Ann Intern Med* 2016; **165**: 305-315 [PMID: 27322798 DOI: 10.7326/M15-1774]

49   **Aithal GP**, Thomas JA, Kaye PV, Lawson A, Ryder SD, Spendlove I, Austin AS, Freeman JG, Morgan L, Webber J. Randomized, placebo-controlled trial of pioglitazone in nondiabetic subjects with nonalcoholic steatohepatitis. *Gastroenterology* 2008; **135**: 1176-1184 [PMID: 18718471 DOI: 10.1053/j.gastro.2008.06.047]

50   **Yau H**, Rivera K, Lomonaco R, Cusi K. The future of thiazolidinedione therapy in the management of type 2 diabetes mellitus. *Curr Diab Rep* 2013; **13**: 329-341 [PMID: 23625197 DOI: 10.1007/s11892-013-0378-8]

51   **Miller ER 3rd**, Pastor-Barriuso R, Dalal D, Riemersma RA, Appel LJ, Guallar E. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Ann Intern Med* 2005; **142**: 37-46 [PMID: 15537682 DOI: 10.7326/0003-4819-142-1-200501040-00110]

52   **Abner EL**, Schmitt FA, Mendiondo MS, Marcum JL, Kryscio RJ. Vitamin E and all-cause mortality: a meta-analysis. *Curr Aging Sci* 2011; **4**: 158-170 [PMID: 21235492 DOI: 10.2174/1874609811104020158]

53   **Vilsbøll T**, Christensen M, Junker AE, Knop FK, Gluud LL. Effects of glucagon-like peptide-1 receptor agonists on weight loss: systematic review and meta-analyses of randomised controlled trials. *BMJ* 2012; **344**: d7771 [PMID: 22236411 DOI: 10.1136/bmj.d7771]

54   **Armstrong MJ**, Gaunt P, Aithal GP, Barton D, Hull D, Parker R, Hazlehurst JM, Guo K; LEAN trial team, Abouda G, Aldersley MA, Stocken D, Gough SC, Tomlinson JW, Brown RM, Hübscher SG, Newsome PN. Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis (LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study. *Lancet* 2016; **387**: 679-690 [PMID: 26608256 DOI: 10.1016/S0140-6736(15)00803-X]

55   **Kim YG**, Hahn S, Oh TJ, Park KS, Cho YM. Differences in the HbA1c-lowering efficacy of glucagon-like peptide-1 analogues between Asians and non-Asians: a systematic review and meta-analysis. *Diabetes Obes Metab* 2014; **16**: 900-909 [PMID: 24655583 DOI: 10.1111/dom.12293]

56   **Cui YM**, Guo XH, Zhang DM, Tham LS, Tang CC, Mace K, Linnebjerg H. Pharmacokinetics, safety, and tolerability of single- and multiple-dose exenatide once weekly in Chinese patients with type 2 diabetes mellitus. *J Diabetes* 2013; **5**: 127-135 [PMID: 23332026 DOI: 10.1111/1753-0407.12020]

57   **Ingwersen SH**, Petri KC, Tandon N, Yoon KH, Chen L, Vora J, Yang W. Liraglutide pharmacokinetics and dose-exposure response in Asian subjects with Type 2 diabetes from China, India and South Korea. *Diabetes Res Clin Pract* 2015; **108**: 113-119 [PMID: 25684604 DOI: 10.1016/j.diabres.2015.01.001]

58   **Cohen DE**, Anania FA, Chalasani N; National Lipid Association Statin Safety Task Force Liver Expert Panel. An assessment of statin safety by hepatologists. *Am J Cardiol* 2006; **97**: 77C-81C [PMID: 16581333 DOI: 10.1016/j.amjcard.2005.12.014]

59   **Kumar S**, Grace ND, Qamar AA. Statin use in patients with cirrhosis: a retrospective cohort study. *Dig Dis Sci* 2014; **59**: 1958-1965 [PMID: 24838495 DOI: 10.1007/s10620-014-3179-2]

60   **Stone NJ**, Robinson JG, Lichtenstein AH, Bairey Merz CN, Blum CB, Eckel RH, Goldberg AC, Gordon D, Levy D, Lloyd-Jones DM, McBride P, Schwartz JS, Shero ST, Smith SC Jr, Watson K, Wilson PW, Eddleman KM, Jarrett NM, LaBresh K, Nevo L, Wnek J, Anderson JL, Halperin JL, Albert NM, Bozkurt B, Brindis RG, Curtis LH, DeMets D, Hochman JS, Kovacs RJ, Ohman EM, Pressler SJ, Sellke FW, Shen WK, Smith SC Jr, Tomaselli GF; American College of Cardiology/American Heart Association Task Force on Practice Guidelines. 2013 ACC/AHA guideline on the treatment of blood cholesterol to reduce atherosclerotic cardiovascular risk in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *Circulation* 2014; **129**: S1-S45 [PMID: 24222016 DOI: 10.1161/01.cir.0000437738.63853.7a]

61   **Flora K**, Hahn M, Rosen H, Benner K. Milk thistle (Silybum marianum) for the therapy of liver disease. *Am J Gastroenterol* 1998; **93**: 139-143 [PMID: 9468229 DOI: 10.1111/j.1572-0241.1998.00139.x]

62   **Wah Kheong C**, Nik Mustapha NR, Mahadeva S. A Randomized Trial of Silymarin for the Treatment of Nonalcoholic Steatohepatitis. *Clin Gastroenterol Hepatol* 2017; **15**: 1940-1949 [PMID: 28419855 DOI: 10.1016/j.cgh.2017.04.016]

63   **Schauer PR**, Bhatt DL, Kirwan JP, Wolski K, Brethauer SA, Navaneethan SD, Aminian A, Pothier CE, Kim ES, Nissen SE, Kashyap SR; STAMPEDE Investigators. Bariatric surgery versus intensive medical therapy for diabetes--3-year outcomes. *N Engl J Med* 2014; **370**: 2002-2013 [PMID: 24679060 DOI: 10.1056/NEJMoa1401329]

64   **Mathurin P**, Hollebecque A, Arnalsteen L, Buob D, Leteurtre E, Caiazzo R, Pigeyre M, Verkindt H, Dharancy S, Louvet A, Romon M, Pattou F. Prospective study of the long-term effects of bariatric surgery on liver injury in patients without advanced disease. *Gastroenterology* 2009; **137**: 532-540 [PMID: 19409898 DOI: 10.1053/j.gastro.2009.04.052]

65   **Lassailly G**, Caiazzo R, Buob D, Pigeyre M, Verkindt H, Labreuche J, Raverdy V, Leteurtre E, Dharancy S, Louvet A, Romon M, Duhamel A, Pattou F, Mathurin P. Bariatric Surgery Reduces Features of Nonalcoholic Steatohepatitis in Morbidly Obese Patients. *Gastroenterology* 2015; **149**: 379-388; quiz e15-16 [PMID: 25917783 DOI: 10.1053/j.gastro.2015.04.014]

66   **Bower G**, Toma T, Harling L, Jiao LR, Efthimiou E, Darzi A, Athanasiou T, Ashrafian H. Bariatric Surgery and Non-Alcoholic Fatty Liver Disease: a Systematic Review of Liver Biochemistry and Histology. *Obes Surg* 2015; **25**: 2280-2289 [PMID: 25917981 DOI: 10.1007/s11695-015-1691-x]

67   **Charlton MR**, Burns JM, Pedersen RA, Watt KD, Heimbach JK, Dierkhising RA. Frequency and outcomes of liver transplantation for nonalcoholic steatohepatitis in the United States. *Gastroenterology* 2011; **141**: 1249-1253 [PMID: 21726509 DOI: 10.1053/j.gastro.2011.06.061]

68   **Nair S**, Verma S, Thuluvath PJ. Obesity and its effect on survival in patients undergoing orthotopic liver transplantation in the United States. *Hepatology* 2002; **35**: 105-109 [PMID: 11786965 DOI: 10.1053/jhep.2002.30318]

69   **Tandon P**, Ney M, Irwin I, Ma MM, Gramlich L, Bain VG, Esfandiari N, Baracos V, Montano-Loza AJ, Myers RP. Severe muscle depletion in patients on the liver transplant wait list: its prevalence and independent prognostic value. *Liver Transpl* 2012; **18**: 1209-1216 [PMID: 22740290 DOI: 10.1002/lt.23495]

70   **Hakeem AR**, Cockbain AJ, Raza SS, Pollard SG, Toogood GJ, Attia MA, Ahmad N, Hidalgo EL, Prasad KR, Menon KV. Increased morbidity in overweight and obese liver transplant recipients: a single-center experience of 1325 patients from the United Kingdom. *Liver Transpl* 2013; **19**: 551-562 [PMID: 23408499 DOI: 10.1002/lt.23618]

MINIREVIEWS

Form confers function: Case of the 3'X region of the hepatitis C virus genome

Mariola Dutkiewicz, Jerzy Ciesiołka

Mariola Dutkiewicz, Jerzy Ciesiołka, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan 61-704, Poland

ORCID number: Mariola Dutkiewicz (0000-0003-1098-0308); Jerzy Ciesiołka (0000-0001-7274-0751).

Abstract

At the 3' end of genomic hepatitis C virus (HCV) RNA there is a highly conserved untranslated region, the 3' X-tail, which forms part of the 3'UTR. This region plays key functions in regulation of critical processes of the viral life cycle. The 3'X region is essential for viral replication and infectivity. It is also responsible for regulation of switching between translation and transcription of the viral RNA. There is some evidence indicating the contribution of the 3'X region to the translation efficiency of the viral polyprotein and to the encapsidation process. Several different secondary structure models of the 3'X region, based on computer predictions and experimental structure probing, have been proposed. It is likely that the 3'X region adopts more than one structural form in infected cells and that a specific equilibrium between the various forms regulates several aspects of the viral life cycle. The most intriguing explanations of the structural heterogeneity problem of the 3'X region came with the discovery of its involvement in long-range RNA-RNA interactions and the potential for homodimer formation. This article summarizes current knowledge on the structure and function of the 3'X region of hepatitis C genomic RNA, reviews previous opinions, presents new hypotheses and summarizes the questions that still remain unanswered.

Key words: Hepatitis C virus; 3'UTR; 3'X-tail; 3'X region; 3'X RNA; RNA structure

Core tip: Several different secondary structure models of the 3'X region have been proposed. It is likely that the 3'X region adopts more than one structural form in infected cells and that a specific equilibrium between the various forms regulates several aspects of the viral life cycle. This article summarizes current knowledge of the structure and function of the 3'X region of hepatitis C genomic RNA,

reviews previous opinions, presents new hypotheses and summarizes the questions that still remain unanswered.

INTRODUCTION

Hepatitis C virus (HCV) belongs to the family *Flaviviridae*, a member of the genus *hepaciviruses*. HCV was identified in 1989 as one of the viruses capable of causing viral hepatitis, in addition to the previously known hepatitis A and B viruses (HAV, HBV)[1]. There are seven major HCV genotypes that differ in virulence and their geographic distribution[2]. Currently, it is estimated that worldwide more than 185 million people are infected with HCV, which represents 2.8% of the world's population[3]. Infection usually takes place in the absence of obvious clinical symptoms, and the resulting inflammation of the liver often progresses to become chronic, usually lasting for years. In time, the chronic inflammation can lead to cirrhosis of the liver and ultimately liver failure or to the development of primary liver cancer[4]. Although the virus replicates mainly in hepatocytes, it also occurs in peripheral blood mononuclear and central nervous system microglia[5,6]. In people with simultaneous infection with HIV (human immunodeficiency virus), HCV replication was also observed in other tissues[7]. Sometimes HCV enters cells of the immune system, leading to very long-lasting effects which are extremely difficult to treat effectively[8].

While progress has been made in the treatment of some other common viral infections, HCV infection remains an important health problem in the world. Current standard treatment methods have the desired therapeutic effect on 40%-60% of patients[4]. The new highly effective drug, grazoprevir, is able to cure patients with 93% effectivity[9], unfortunately, the treatment is still very expensive and out of reach for the majority of the infected individuals, many of whom live in Third World countries. So far, no HCV vaccine has been developed, which is due to the high genetic variability of the virus, comparable to the genetic variability of HIV. In the absence of widely accessible conventional drugs and vaccines, numerous attempts have been made to design inhibitors of viral proteins, inhibitory oligomers of the antisense and ribozyme type, and more recently also of RNA interference tools directed against viral RNA[4,10,11].

HCV is a small, enveloped virus with a diameter of 40-60 nm, whose genome is a single-stranded, positive-sense RNA of about 9.6 kb in length[6,10,12]. In the viral replication process, the positive-sense RNA strand is transcribed into negative-sense counterpart, the replication intermediate, which serves as the template for the RNA synthesis of progeny genomes. The HCV genome has one very long open reading frame (ORF). It encodes a precursor polyprotein, which is digested in a series of cleavage processes to finally produce proteins: C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B[13]. The coding sequence of the HCV genome is flanked by two untranslated regions: the 5'UTR and the 3'UTR (5' and 3' untranslated regions). Both these regions play key functions in regulating HCV life cycle and determine its expression level. The 5'UTR contains a highly structured regulatory element, an IRES (internal ribosome entry site), that enables cap-independent translation. The 3' UTR is engaged in the replication process and in the regulation of translation. In addition, control of other processes as, for example, assembly of virions and switching between different developmental phases takes place with the participation of the structural RNA elements present at the very 3' end of the HCV genome.

This article presents the current state of knowledge about the structure and functions of the most terminal section of the 3'UTR of hepatitis C virus, the 3'X-tail.

STRUCTURE OF THE 3'UTR REGION

HCV 3'UTR has a variable length of 170 to 250 nucleotides (Figure 1). Three characteristic sections have been recognized in this region: immediately after the stop codon there is a variable region about 25-130 nucleotides in length, characterized by high sequence heterogeneity between various genotypes, but conserved within the same genotype of the virus. Next there is a poly-pyrimidine segment of varying length, independent of the type, or even a subtype of virus, ranging from about 30 to 130 nucleotides. At the very end of the genome, there is an 3'X region, discovered 6 years after cloning of the virus, which is 98 nucleotides long and is an almost absolutely conserved sequence[6].

Within the first, variable region of 3'UTR, two sequence motifs are present that are found in all genotypes of HCV. These are: the ACACUCC section, which represents a seed-region for miR-122[14], and the UG dinucleotide located at the very end of the region, directly upstream the poly (U/UC) section[6]. The stop codon is located in the apical loop of the stem-loop motif, named 5BSL3.4 or SL9360, which is created partly from the terminal nucleotides of the ORF encoding the NS5B protein and partly from non-coding nucleotides. The poly-pyrimidine section can be divided into a two poly (UC) parts uneven in length, which are separated by the one poly (U) region. The poly-pyrimidine segment is heterogeneous, not only in terms of length, but also in nucleotide sequence. In genotypes 2a, 3a, 3b there are several conserved adenosine residues in this region that are missing in genotypes 1b and 2b[6]. Individual guanosine residues are also observed on rare occasions.

3'X-tail

Initially, it was suspected that at the 3' terminus of the HCV genome a poly (U) or poly (A) sequence was
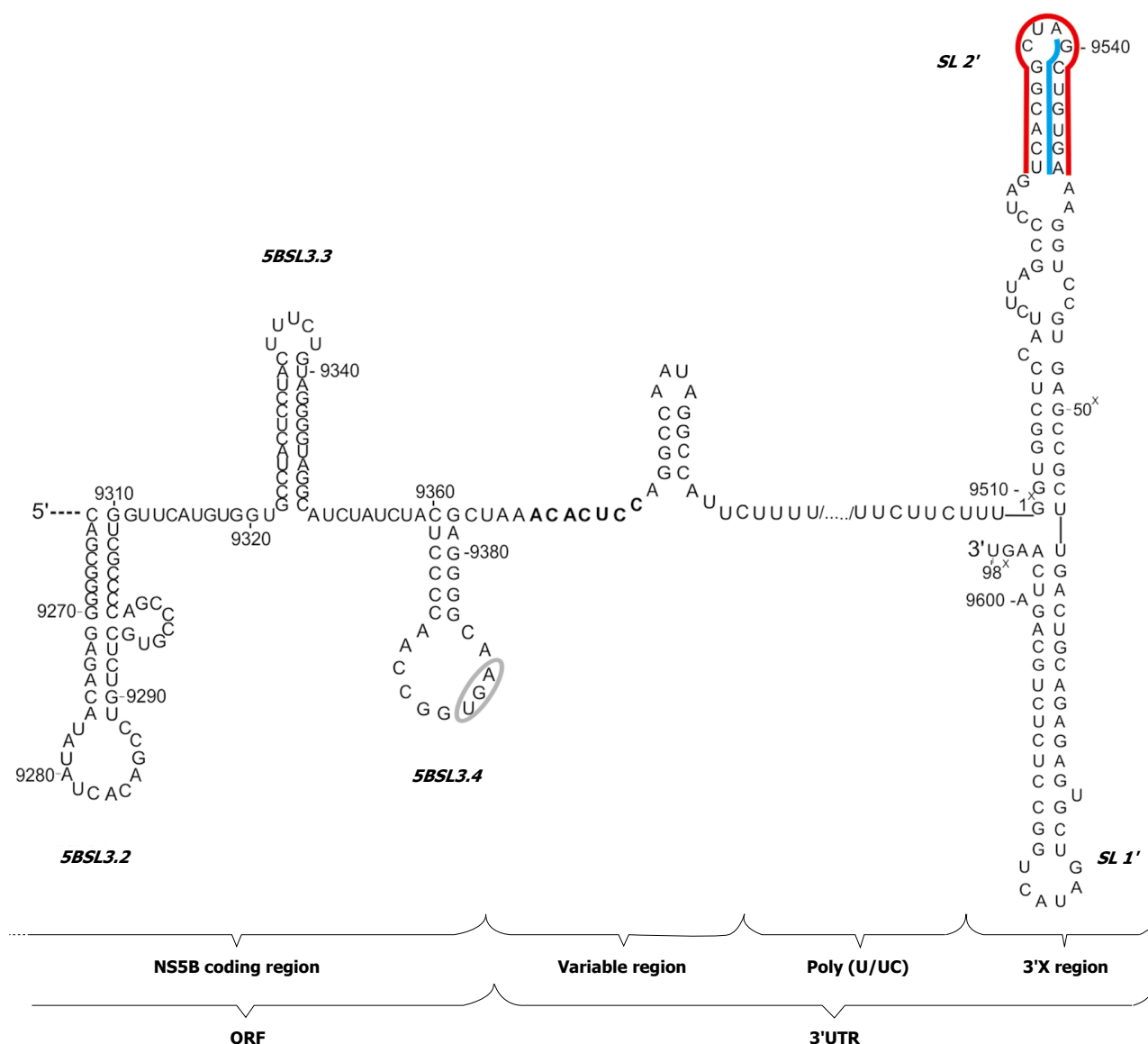
Figure 1 Secondary structure model of the 3'UTR of hepatitis C virus genome with adjacent 3' terminal sequence of the coding region. The stop codon is indicated by a gray ellipse. The regions involved in the kissing-interactions are marked with blue lines; the region involved in dimerization is indicated by a red line; the seed region for miR122 is highlighted in bold.

present. The existence of the 98-nt long 3'X region was discovered by Tanaka and colleagues in 1995[15]. Almost simultanously the presence of the 3'X at the end of the HCV genome identified Kolykhalov *et al*[16]. Comparison of the sequence of this RNA segment in different viral isolates indicated 96%-100% sequence conservation of the 3'X region, with only single substitutions in the 3' terminal 46-nt sequence[6]. This is an unusual feature for this dynamically changing virus, that suggests its extremely important function.

Several different secondary structure models of the 3'X region were proposed, based on computer predictions and experimental structure probing by chemical modification, enzyme digestion, RNA cleavage induced by Pb^{2+} ions, NMR (nuclear magnetic resonance) and SAXS (small angle X-ray scattering). A stable structure of the SL1 hairpin was proposed for the 3' part of the 3'X RNA, which is common to different structural models (Figure 2)[17-21]. The 52-nucleotide segment making up the 5' part of the 3'X region could not be assigned an unambiguous structure based on the experimental results obtained. The proposed models were only partially confirmed by the results of experimental studies[6,17,18]. Poor ordering of this fragment or formation of more than one structural form have been suggested[17,18,20].

One of the first structural models of the 3'X region suggested the presence of three hairpin motifs: SL1, SL2, and SL3 (Figure 2A)[17,18]. Another structural model suggested a set of four hairpins: SL1, SL2a, SL2b and SL3, where SL1 and SL3 did not differ from the first model, but two shorter hairpins replaced the SL2 motif[20]. The main reason for the proposed change was the observation of strong DMS modifications, as well as Pb^{2+} ion-induced cleavages, in the middle of the SL2 double-stranded stem (C44-C45), which indicated a single-stranded or highly flexible region there. In this four

Figure 2 Diverse secondary structure models proposed for the 3'X region of hepatitis C virus genome. A: The 3xSL model[17,18]; B: The 4xSL model[20]; C: The 2xSL model[21]. The region involved in the kissing-interactions is indicated with blue line, the region involved in dimerization is marked with red line; possible alternative folding of separate fragments are displayed as gray rectangles.

stem-loop model (4xSL), the reactive cytidine residues are located in the apical loop of the short SL2b motif (Figure 2B). In addition, it was noted that the SL2a and SL2b hairpins have the potential to create a pseudoknot after rearrangement of their base pairing[20]. However, discovery of functionally important long-range kissing interactions between a sequence located in the apical loop of the SL2 hairpin and the upstream sequence in the region encoding NS5B[22] (see next section), seemed to support the three- (3xSL), but not the four- (4xSL) stem-loop model for the 3'X region. Thus, the 3xSL structure model of the 3'X-tail became the favored idea during the following decade.

Recently, based on NMR and SAXS studies, a two-hairpin model (2xSL) has been proposed for the 3'X region, consisting of the SL1 and SL2' (named elsewhere also SL2/3) (Figure 2C)[21,23,24]. In addition, in this model the SL1 hairpin may fold in two different ways: the closed structure - SL1 or the open one - SL1', differing by the three terminal base-pairs of the hairpin being paired or unpaired, respectively (Figure 2C)[21,23,25]. In the 2xSL model the previously identified flexible cytosine residues (C44-C45) are located within the internal loop of the SL2' motif. The closed conformation of SL1 is associated with long-range kissing interactions with another part of the

genome, while the open conformation, SL1' with the 3' overhang is associated with dimerization (described in the next section).

The role of selected metal ions was investigated regarding its influence on the structure of the 3'X-tail but it seems that neither magnesium, nor sodium ion concentration determine its folding, within the range of normal physiological conditions[26]. However, at higher ionic strength, extended homodimers are preferentially formed over 2xSL monomers[24]. A chaperone role of the viral C protein (core protein), has also been suggested[23,27]. Long-range RNA-RNA interactions with 5' sequences in the genome or with a second genomic RNA molecule seems to influence the structure of the 3'X region more than the presence of specific metal ions.

It is very likely that the 3'X region can adopt more than one structural form in infected cells and that a specific equilibrium between these forms regulates several processes of the viral life cycle. These different structural forms may be favored by distinct viral genotypes what can help to explain their differential virulence and drug resistance[28,29].

Long range RNA-RNA interactions
Previous investigation of genomic HCV RNA and a con-

struct containing only 5'UTR and 3'UTR did not show any interaction of the X region with other regions of the molecules studied. This suggested the structural independence of these two regions of viral RNA from each other[17]. Later tertiary[30-32] interactions at the 3' end of the HCV genomic strand were proposed by Friebe *et al*[22]. The kissing interactions between the absolutely conserved "k" segment of the X region: 32[X]-GCUGUGA-38[X] and the "k'" segment of the NS5B coding sequence: 9281-UCACAGC-9287 do not require a protein chaperone. These sequence stretches are located within apical parts of SL elements called SL2 or SL2' and 5BSL3.2, which is one of the domains in the CRE (*cis*-acting replication element) (Figure 1)[22,28,31,33]. It is easy to imagine that kissing interactions could be initiated by any complementary stretch of nucleotides located within two apical loops of RNA hairpins. This scenario was previously suggested for the 3xSL model of the 3'X-tail[22,28,31,33]. However, recent studies with the use of mutagenesis, NMR and SAXS methods, indicate that before kissing-interactions are formed, the "k"- sequence in the X region is involved in base pairing within the SL2' element[21,24]. How the 5BSL3.2 element is able to induce the conformational transition from SL2' to SL2, or more globally: from 2xSL to 3xSL form of the 3'X, remains unclear. The next great challenge is to elucidate step by step how this transition occurs.

Two replicon systems, Con1b and JF-H1, have been investigated with a SHAPE method, which is based on a chemical modification of single-stranded RNA residues. The replicons are constructed on the basis of two different viral genotypes 1b and 2a, respectively. The experimental results showed that the proposed kissing interactions were detectable only for replicon JF-H1 (genotype 2a). The results obtained for replicon Con1b were in agreement with data obtained for the genotype 1a (strain H) of the virus and favors the open conformation of SL1'. In this open conformation the very 3' end of the SL' remains single-stranded, and that is associated with an increase in the efficiency of RNA synthesis initiation. In fact, subtype 1b is more virulent and resistant to interferon-based therapy than other genotypes, including subtype 2a[29,34]. This suggests how virus virulence and drug response is significantly influenced by the long-range kissing interactions, which likely cause changes in the base-pairing character of the very 3' terminal nucleotides.

Dimerization

Another intriguing set of tertiary interactions were proposed for the 3'X region of HCV genome by Ivanyi-Nagy *et al*[23]. Primarily *in vitro* investigations showed that the apical part of the SL2 hairpin in the 3xSL model, 29[X]-CUAG-32[X], is able to interact with the respective palindromic sequence in the second 3'X RNA molecule thus inducing the formation of a homodimer[35]. The 16-nt palindromic sequence, called also DLS (dimerization leading stretch) is absolutely conserved among all HCV genotypes[36]. The homodimer, consisting of two isolated 3'X-RNA molecules, was characterized *in vitro* by NMR and SAXS. It was proposed that the resulting homoduplex could involve shorter (SL2) or extended (SL2') sequence fragments (Figure 3)[21,23-25,35]. The core protein supports the formation and stabilizes the extended homodimer[24].

The dimerization of the HCV genome still remains unproved *in vivo* and its function remains to be elucidated. It was suggested that the dimerization could be helpful in ensuring that only full-length progeny RNA molecules are encapsidated[24]. Moreover, the unwinding of the 3'-end of the genome, could greatly facilitate the minus RNA synthesis[37]. Masante *et al*[38] suggested that the homodimeric genome operates as a preferred template for the HCV polymerase (NS5B). Additionally, dimerization might enhance the rate of RNA recombination between two homologue RNA strands (resumed in[39]). The equilibrium between 2xSL monomers and dimers would likely also be tuned by the local concentration of RNA and the presence of core protein[24].

FUNCTIONS OF THE 3'UTR REGION

Hepatitis C virus can only infect humans and chimpanzees; there is no experimental model of its infectivity among small animals. For this reason, very few studies on the spread of the virus have been carried out *in vivo*. An experiment was carried out by Yanagi *et al*[40], in which a number of viral constructs containing deletions within the 3'UTR were injected into the liver of the chimpanzee at time intervals, and then the animal was examined for the presence of HCV RNA, anti-HCV antibodies and liver enzymes in its serum. Viral mutants lacking the entire 3'X region or parts thereof (nt: 1-50 and 57-98) were not able to replicate. Also viral infection was not observed when a mutant containing no poly (U/UC) segment was used. Only the construct devoid of 24 nucleotides within the variable region turned out to be infectious, indicating that this region is not essential for the viral life cycle under these conditions[40]. A similar experiment was carried out by Kolykhalov *et al*[41], and the obtained results were in line with previous observations of Yanagi *et al*[40].

One of the possible explanations of 3'X region function was its influence on genomic RNA stability. This has been shown *in vitro* but is not equally important for all genotypes of the virus[42].

Replication process

The information on the involvement of individual parts of the 3'UTR in the life cycle of the virus presented in the previous section was confirmed in research with the replicating Huh7 and HeLa cell systems[19,22,42,43]. Namely, constructs devoid of the entire poly (U/UC) section, were not able to replicate[42,43], and the minimum length of the polypyrimidine segment was 50 nt in one study[43], and only 26 nt in the other[42]. In contrast, the deletion of the
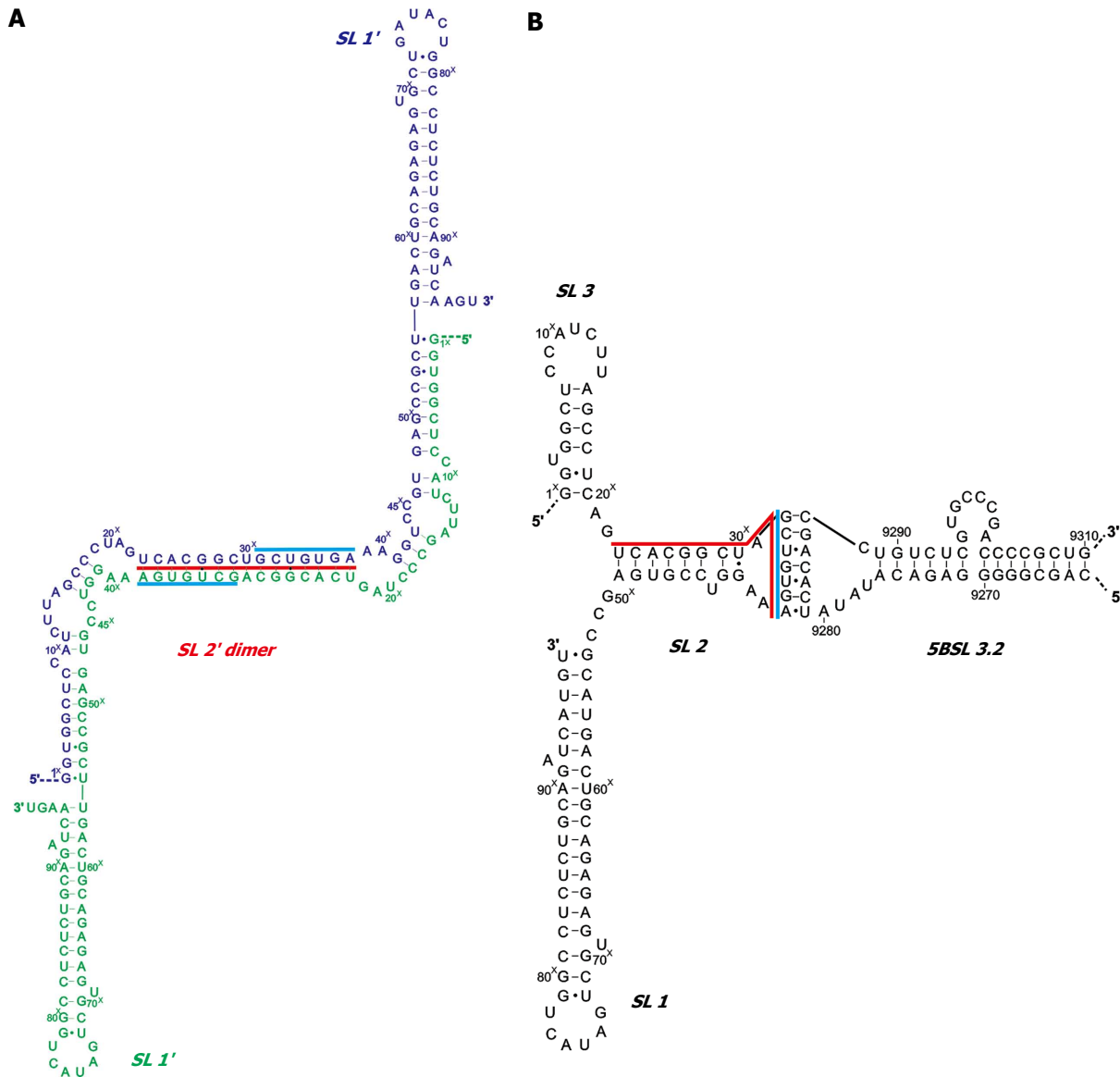
Figure 3 Long-range RNA-RNA interactions proposed for the 3'X region of hepatitis C virus genome. A: The homodimeric interactions between two 3'X regions embedded into two RNA molecules, model according to Cantero-Camacho *et al*[24]; B: The kissing-interactions with SL5B3.2[22,28,31,33]. Nucleotide sequence involved in dimerization is additionally indicated with red line, while those involved in the kissing-interactions are marked with blue lines.

3'X region or any one of its parts, SL1, SL2 or SL3, led to a complete failure to replicate[42,43] and only few point mutations in this region were tolerated[19,43]. In turn, the removal of the variable region from the 3'UTR of the viral genome only reduced the efficiency of the process leading to decrease in the rate of replication[22,42,43]. In addition, it has been shown that the region directly upstream the stop codon plays a key role in viral replication[22].

The site of the NS5B polymerase attachment within region 3'X has been mapped to be within the SL2 sequence and within the SL1[44]. They are protected against digestion with RNase T1 at guanosine residues at positions 41, 42, 50 and 53. The direct interaction of the 3'X RNA with the NS5B protein has been found in studies conducted *in vitro*[6,12,44-46]. The specificity of the viral polymerase to the model RNA template is relatively

low and the presence of the 3'X region is not always necessary for RNA synthesis[6,12,37,45]. However, in the case of matrices containing genomic or subgenomic RNA, the presence of the 3'X region is necessary for the efficiency and the specificity of the process. It has been shown that the lack of this sequence, or a part thereof, almost completely inhibits replication, and in the case of deletion of nucleotides in positions 31-40, the product is too long[12,44].

The NS5B polymerase catalytic center probably only interacts with a single-stranded RNA fragment. The initiation of replication seems to take place in the SL1 loop, 21 nucleotides from the 3' end of the 3'X RNA[12]. However, another research group, Kim *et al*[46] indicates that the process begins near the 3' terminus, in a region rich in purines. However, in studies carried out in a cellular system, it has been shown that the presence

of the 3' terminal GU dinucleotide is preferred in the reaction[19]. Similar preferences of the replicase for the U at the 3'-end of the template RNA were observed by Shimm *et al*[47] in the *in vitro* system. Also, Kao *et al*[37] postulate that the enzyme requires a stable secondary template structure and at least one unpaired cytidine residue at its 3' end to initiate RNA synthesis. Butcher *et al*[48] proposed a replication initiation scenario, one common to various polymerases, in which the synthesis of a new strand of RNA begins with a nucleotide complementary to the penultimate of the 3' end of the nucleotide of the template molecule. Secondly, a complementary nucleotide is added to the remainder of the template at the 3' end, and only after the synthesis of this dinucleotide-primer is the complementary strand of viral RNA synthesized. Many observations suggest that NS5B is a non-specific enzyme, *i.e.* it can recognize more than one nucleotide sequence. The presence of specific Ⅰ-, Ⅱ- and tertiary structures at the 3'-end of the template RNA may allow modulation of the specificity of the enzyme for better yield and/or greater precision in the selection of the origin. In addition, both viral and host proteins can be involved in the replication process[6,49].

In summary, it should be noted that this replication phase of the life cycle of the virus has not yet been precisely understood. In addition to interacting with the viral polymerase, the 3'X region also interacts with other proteins one of them being the NS3 viral protein[49]. Acting as a helicase, it is probably a very important component of the replication complex, because the NS5B polymerase tends to detach from the RNA template when it encounters very stable RNA secondary structures[50]. The specific interaction between NS3 and the HCV 3'UTR probably involves a large part of this region containing the sequence 3'X and the poly (U/UC) section, since none of these elements alone is sufficient to form a stable complex with this protein[49].

Translation process

One of the earliest known cellular proteins interacting with HCV 3'UTR was the PTB protein. The interaction of PTB with the 3'X region may be related to the regulation of the translation process. The proposed site of PTB binding is 21 nucleotides from the 5' end of the 3'X region (SL3), containing a part of the consensus sequence recognized by this protein[18]. Similar results are presented by Tsuchihara *et al*[51] suggesting that the region involved in the interaction with PTB is 19 nucleotides from the 5' end of the 3'X region and extends 7 nucleotides upstream this sequence. The mutagenesis data showed that both secondary structure and the nucleotide sequence in the SL2 and SL3 regions are important for this interaction[18,51].

The 3'UTR has been ascribed as a translation enhancer[52-56]. One of mechanisms proposed by which enhancing the process is achieved, is the interaction between the 5' and 3' ends of the HCV genome, mediated by the PTB protein and another, hypothetical Y

protein[57]. The effect of enhancing translation through genome cyclization is observed in many viruses[58]. However, not all results indicate PTB enhances HCV translation, sometimes the protein seems to be even inhibitory[59]. There is also no consensus on the impact of the 3'UTR on the efficiency of translation. For example, Murakami *et al*[59] observed that in an *in vitro* system, deletion of SL3 in the 3'X region and/or the poly (U/UC) section resulted in an increase in the amount of protein produced. A possible explanation for this observation would be that these sequence fragments, by interacting with other protein factors, inhibit the translation process. In other studies, however, it was observed that the presence of 3'UTR had no effect on the increase of the product amount and the efficiency of the polyprotein cleavage process[43,60]. It appears the choice of the experimental model plays a key role in this kind of research. For example, while in the lysate from rabbit reticulocytes no effect of 3'UTR on translation efficiency is observed[42,53], in HeLa cells, hepatocytes and in an *in vivo* mouse model the presence of the 3'UTR stimulates this process[53]. Recently, the role of 3'UTR in enhancing translational efficiency has been reported both in the rabbit reticulocyte lysate and in Huh7 cells[61].

Finally, it has been shown that the region X interacts with the ribosomal proteins L22, L3, S3 and mL3[62]. Similar viral RNA - L22 protein interactions have been observed for EBV (Epstein-Barr virus) and HPV-1 (human type 1 papilloma virus), showing a positive effect of L22 on translational efficiency[62]. Meanwhile, in the case of Qβ virus, ribosomal host proteins are involved in the formation of the viral replication complex[62]. It is unclear whether the interaction of ribosomal proteins with the HCV X region is related to the replication or translation process, or to both of these processes. The demonstrated ability of HCV protein NS5B to bind to the ribosome[63] is reminiscent of the strategy used by the Qβ virus, which uses ribosomal proteins to build its replication complex.

CONCLUSION

The most intriguing feature of the 3'X region is its extremely high sequence conservation. The maintenance of a 98-nt long stretch of RNA at over 95% conservation places severe constraints on such a fast-mutating RNA virus. Apparently almost any change to this stretch result in progeny incapable of reproducing which rapidly disappear from the population.

The high sequence conservation might be explained by existence of different structural forms of the 3'X region. Within one structure there is usually a way to neutralize point mutations by adaptive mutations in another structure, thus restoring important base-pairing. Simultaneous compensation of mutations in two (or more) different structures/forms is much less probable. However, instead of several different structural forms of the X-region, the long-range RNA-RNA interactions with the involvement of that region could explain its high

sequence conservation.

Evidence indicates the 3'X region is involved in a number of interactions including kissing 5BSL3.2 (required for replication), an interaction with the second RNA molecule carrying the X-region sequence (homo-dimerization), binding of the NS5B protein (for initiation of RNA synthesis), an interaction with NS3 helicase (for elongation of RNA synthesis), binding of PTB (for proposed translation regulation). Is it possible that one structural form of the 3'X region supports such different interactions? The answer is: yes, it is possible, but unlikely. On the other hand, it is easy to imagine that different structural forms of this region are responsible for different processes and interactions. For instance: 2xSL - structure for dimerization and enhanced RNA synthesis, 3xSL - structure for kissing interactions that supports translation.

Hopefully in-depth *in vivo* structure mapping of the 3'X-region at the various replication stages, in different cellular compartments will answer these questions to give us a better understanding of the virus and lead to additional means to control it.

ACKNOWLEDGMENTS

REFERENCES

1   **Choo KB**, Liew LN, Wang HC. Production of transgenic mice carrying the human hepatitis B virus or the human papillomavirus DNA sequences in Taiwan: analysis of physical structure and hereditary mode. *Proc Natl Sci Counc Repub China B* 1989; **13**: 314-318 [PMID: 2561574]

2   **Bartenshlager R**. Hepatitis C Virus: From Molecular Virology to Antiviral Therapy. *Current Topics in Microbiology and Immunology* 2013; **369**: V-Ⅵ [DOI: 10.1007/978-3-642-27340-7]

3   **Mohd Hanafiah K**, Groeger J, Flaxman AD, Wiersma ST. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. *Hepatology* 2013; **57**: 1333-1342 [PMID: 23172780 DOI: 10.1002/hep.26141]

4   **Dev A**, Patel K, McHutchison JG. New therapies for chronic hepatitis C virus infection. *Curr Gastroenterol Rep* 2004; **6**: 77-86 [PMID: 14720458 DOI: 10.1007/s11894-004-0030-5]

5   **Rijnbrand RC**, Lemon SM. Internal ribosome entry site-mediated translation in hepatitis C virus replication. *Curr Top Microbiol Immunol* 2000; **242**: 85-116 [PMID: 10592657 DOI: 10.1007/978-3-642-59605-6_5]

6   **Shi ST**, Lai MM. Hepatitis C viral RNA: challenges and promises. *Cell Mol Life Sci* 2001; **58**: 1276-1295 [PMID: 11577984 DOI: 10.1007/PL00000939]

7   **Laskus T**, Radkowski M, Wang LF, Vargas H, Rakela J. The presence of active hepatitis C virus replication in lymphoid tissue in patients coinfected with human immunodeficiency virus type 1. *J Infect Dis* 1998; **178**: 1189-1192 [PMID: 9806058 DOI: 10.1086/515682]

8   **Conca P**, Cafaro G, De Renzo A, Coppola A, Cimino E, Tarantino G. Successful and Safe Long-Term Standard Antiviral Therapy in a Patient with „Explosive" Immune Response in Course of HCV-Related Liver Cirrhosis. *Int J Mol Sci* 2015; **16**: 14075-14085 [PMID: 26101866 DOI: 10.3390/ijms160614075]

9   **Bell AM**, Wagner JL, Barber KE, Stover KR. Elbasvir/Grazoprevir: A Review of the Latest Agent in the Fight against Hepatitis C. *Int J Hepatol* 2016; **2016**: 3852126 [PMID: 27403342 DOI: 10.1155/2016/3852126]

10   **Gómez J**, Nadal A, Sabariegos R, Beguiristain N, Martell M, Piron M. Three properties of the hepatitis C virus RNA genome related to antiviral strategies based on RNA-therapeutics: variability, structural conformation and tRNA mimicry. *Curr Pharm Des* 2004; **10**: 3741-3756 [PMID: 15579068 DOI: 10.2174/1381612043382675]

11   **Randall G**, Rice CM. Interfering with hepatitis C virus RNA replication. *Virus Res* 2004; **102**: 19-25 [PMID: 15068876 DOI: 10.1016/j.virusres.2004.01.011]

12   **Oh JW**, Ito T, Lai MM. A recombinant hepatitis C virus RNA-dependent RNA polymerase capable of copying the full-length viral RNA. *J Virol* 1999; **73**: 7694-7702 [PMID: 10438859]

13   **Penin F**, Dubuisson J, Rey FA, Moradpour D, Pawlotsky JM. Structural biology of hepatitis C virus. *Hepatology* 2004; **39**: 5-19 [PMID: 14752815 DOI: 10.1002/hep.20032]

14   **Gerresheim GK**, Dünnes N, Nieder-Röhrmann A, Shalamova LA, Fricke M, Hofacker I, Höner Zu Siederdissen C, Marz M, Niepmann M. microRNA-122 target sites in the hepatitis C virus RNA NS5B coding region and 3' untranslated region: function in replication and influence of RNA secondary structure. *Cell Mol Life Sci* 2017; **74**: 747-760 [PMID: 27677491 DOI: 10.1007/s00018-016-2377-9]

15   **Tanaka T**, Kato N, Cho MJ, Shimotohno K. A novel sequence found at the 3' terminus of hepatitis C virus genome. *Biochem Biophys Res Commun* 1995; **215**: 744-749 [PMID: 7488017 DOI: 10.1006/bbrc.1995.2526]

16   **Kolykhalov AA**, Feinstone SM, Rice CM. Identification of a highly conserved sequence element at the 3' terminus of hepatitis C virus genome RNA. *J Virol* 1996; **70**: 3363-3371 [PMID: 8648666]

17   **Blight KJ**, Rice CM. Secondary structure determination of the conserved 98-base sequence at the 3' terminus of hepatitis C virus genome RNA. *J Virol* 1997; **71**: 7345-7352 [PMID: 9311812]

18   **Ito T**, Lai MM. Determination of the secondary structure of and cellular protein binding to the 3'-untranslated region of the hepatitis C virus RNA genome. *J Virol* 1997; **71**: 8698-8706 [PMID: 9343228]

19   **Yi M**, Lemon SM. Structure-function analysis of the 3' stem-loop of hepatitis C virus genomic RNA and its role in viral RNA replication. *RNA* 2003; **9**: 331-345 [PMID: 12592007 DOI: 10.1261/rna.2144203]

20   **Dutkiewicz M**, Ciesiolka J. Structural characterization of the highly conserved 98-base sequence at the 3' end of HCV RNA genome and the complementary sequence located at the 5' end of the replicative viral strand. *Nucleic Acids Res* 2005; **33**: 693-703 [PMID: 15681619 DOI: 10.1093/nar/gki218]

21   **Cantero-Camacho Á**, Gallego J. The conserved 3'X terminal domain of hepatitis C virus genomic RNA forms a two-stem structure that promotes viral RNA dimerization. *Nucleic Acids Res* 2015; **43**: 8529-8539 [PMID: 26240378 DOI: 10.1093/nar/gkv786]

22   **Friebe P**, Boudet J, Simorre JP, Bartenschlager R. Kissing-loop interaction in the 3' end of the hepatitis C virus genome essential for RNA replication. *J Virol* 2005; **79**: 380-392 [PMID: 15596831 DOI: 10.1128/jvi.79.1.380-392.2005]

23   **Ivanyi-Nagy R**, Kanevsky I, Gabus C, Lavergne JP, Ficheux D, Penin F, Fossé P, Darlix JL. Analysis of hepatitis C virus RNA dimerization and core-RNA interactions. *Nucleic Acids Res* 2006; **34**: 2618-2633 [PMID: 16707664 DOI: 10.1093/nar/gkl240]

24   **Cantero-Camacho Á**, Fan L, Wang YX, Gallego J. Three-dimensional structure of the 3'X-tail of hepatitis C virus RNA in monomeric and dimeric states. *RNA* 2017; **23**: 1465-1476 [PMID: 28630140 DOI: 10.1261/rna.060632.117]

25   **Cristofari G**, Ivanyi-Nagy R, Gabus C, Boulant S, Lavergne JP, Penin F, Darlix JL. The hepatitis C virus Core protein is a potent nucleic acid chaperone that directs dimerization of the viral (+) strand RNA in vitro. *Nucleic Acids Res* 2004; **32**: 2623-2631 [PMID: 15141033 DOI: 10.1093/nar/gkh579]

26   **Kranawetter C**, Brady S, Sun L, Schroeder M, Chen SJ, Heng X. Nuclear Magnetic Resonance Study of RNA Structures at the 3'-End of the Hepatitis C Virus Genome. *Biochemistry* 2017; **56**: 4972-4984 [PMID: 28829576 DOI: 10.1021/acs.biochem.7b00573]

27   **Sharma KK**, de Rocquigny H, Darlix JL, Lavergne JP, Pénin F,

Lessinger JM, Mély Y. Analysis of the RNA chaperoning activity of the hepatitis C virus core protein on the conserved 3'X region of the viral genome. *Nucleic Acids Res* 2012; **40**: 2540-2553 [PMID: 22127859 DOI: 10.1093/nar/gkr1140]

28 **Tuplin A**, Struthers M, Simmonds P, Evans DJ. A twist in the tail: SHAPE mapping of long-range interactions and structural rearrangements of RNA elements involved in HCV replication. *Nucleic Acids Res* 2012; **40**: 6908-6921 [PMID: 22561372 DOI: 10.1093/nar/gks370]

29 **Carter LDB**, Aronsohn A. Overcoming injustice: A roadmap to improve access to hepatitis C virus therapy for our medicaid patients. *Hepatology* 2017; **65**: 1735-1740 [PMID: 28160311 DOI: 10.1002/hep.29095]

30 **Diviney S**, Tuplin A, Struthers M, Armstrong V, Elliott RM, Simmonds P, Evans DJ. A hepatitis C virus cis-acting replication element forms a long-range RNA-RNA interaction with upstream RNA sequences in NS5B. *J Virol* 2008; **82**: 9008-9022 [PMID: 18614633 DOI: 10.1128/JVI.02326-07]

31 **You S**, Rice CM. 3' RNA elements in hepatitis C virus replication: kissing partners and long poly(U). *J Virol* 2008; **82**: 184-195 [PMID: 17942554 DOI: 10.1128/JVI.01796-07]

32 **You S**, Stump DD, Branch AD, Rice CM. A cis-acting replication element in the sequence encoding the NS5B RNA-dependent RNA polymerase is required for hepatitis C virus RNA replication. *J Virol* 2004; **78**: 1352-1366 [PMID: 14722290 DOI: 10.1128/jvi.78.3.1352-1366.2004]

33 **Tuplin A**, Struthers M, Cook J, Bentley K, Evans DJ. Inhibition of HCV translation by disrupting the structure and interactions of the viral CRE and 3' X-tail. *Nucleic Acids Res* 2015; **43**: 2914-2926 [PMID: 25712095 DOI: 10.1093/nar/gkv142]

34 **Irshad M**, Mankotia DS, Irshad K. An insight into the diagnosis and pathogenesis of hepatitis C virus infection. *World J Gastroenterol* 2013; **19**: 7896-7909 [PMID: 24307784 DOI: 10.3748/wjg.v19.i44.7896]

35 **Shetty S**, Kim S, Shimakami T, Lemon SM, Mihailescu MR. Hepatitis C virus genomic RNA dimerization is mediated via a kissing complex intermediate. *RNA* 2010; **16**: 913-925 [PMID: 20360391 DOI: 10.1261/rna.1960410]

36 **Yamada N**, Tanihara K, Takada A, Yorihuzi T, Tsutsumi M, Shimomura H, Tsuji T, Date T. Genetic organization and diversity of the 3' noncoding region of the hepatitis C virus genome. *Virology* 1996; **223**: 255-261 [PMID: 8806561 DOI: 10.1006/viro.1996.0476]

37 **Kao CC**, Yang X, Kline A, Wang QM, Barket D, Heinz BA. Template requirements for RNA synthesis by a recombinant hepatitis C virus RNA-dependent RNA polymerase. *J Virol* 2000; **74**: 11121-11128 [PMID: 11070008 DOI: 10.1128/JVI.74.23.11121-11128.2000]

38 **Masante C**, Jaubert C, Palau W, Plissonneau J, Besnard L, Ventura M, Di Primo C. Mutations of the SL2 dimerization sequence of the hepatitis C genome abrogate viral replication. *Cell Mol Life Sci* 2015; **72**: 3375-3385 [PMID: 25822205 DOI: 10.1007/s00018-015-1893-3]

39 **Romero-López C**, Barroso-delJesus A, Berzal-Herranz A. The chaperone-like activity of the hepatitis C virus IRES and CRE elements regulates genome dimerization. *Sci Rep* 2017; **7**: 43415 [PMID: 28233845 DOI: 10.1038/srep43415]

40 **Yanagi M**, St Claire M, Emerson SU, Purcell RH, Bukh J. In vivo analysis of the 3' untranslated region of the hepatitis C virus after in vitro mutagenesis of an infectious cDNA clone. *Proc Natl Acad Sci U S A* 1999; **96**: 2291-2295 [PMID: 10051634 DOI: 10.1073/pnas.96.5.2291]

41 **Kolykhalov AA**, Mihalik K, Feinstone SM, Rice CM. Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3' nontranslated region are essential for virus replication in vivo. *J Virol* 2000; **74**: 2046-2051 [PMID: 10644379 DOI: 10.1128/JVI.74.4.2046-2051.2000]

42 **Yi M**, Lemon SM. 3' nontranslated RNA signals required for replication of hepatitis C virus RNA. *J Virol* 2003; **77**: 3557-3568 [PMID: 12610131 DOI: 10.1128/JVI.77.6.3557-3568.2003]

43 **Friebe P**, Bartenschlager R. Genetic analysis of sequences in the 3' nontranslated region of hepatitis C virus that are important for RNA replication. *J Virol* 2002; **76**: 5326-5338 [PMID: 11991961 DOI: 10.1128/JVI.76.11.5326-5338.2002]

44 **Oh JW**, Sheu GT, Lai MM. Template requirement and initiation site selection by hepatitis C virus polymerase on a minimal viral RNA template. *J Biol Chem* 2000; **275**: 17710-17717 [PMID: 10749880 DOI: 10.1074/jbc.M908781199]

45 **Reigadas S**, Ventura M, Sarih-Cottin L, Castroviejo M, Litvak S, Astier-Gin T. HCV RNA-dependent RNA polymerase replicates in vitro the 3' terminal region of the minus-strand viral RNA more efficiently than the 3' terminal region of the plus RNA. *Eur J Biochem* 2001; **268**: 5857-5867 [PMID: 11722573 DOI: 10.1046/j.0014-2956.2001.02532.x]

46 **Kim M**, Kim H, Cho SP, Min MK. Template requirements for de novo RNA synthesis by hepatitis C virus nonstructural protein 5B polymerase on the viral X RNA. *J Virol* 2002; **76**: 6944-6956 [PMID: 12072495 DOI: 10.1128/JVI.76.14.6944-6956.2002]

47 **Shim JH**, Larson G, Wu JZ, Hong Z. Selection of 3'-template bases and initiating nucleotides by hepatitis C virus NS5B RNA-dependent RNA polymerase. *J Virol* 2002; **76**: 7030-7039 [PMID: 12072503 DOI: 10.1128/JVI.76.14.7030-7039.2002]

48 **Butcher SJ**, Grimes JM, Makeyev EV, Bamford DH, Stuart DI. A mechanism for initiating RNA-dependent RNA polymerization. *Nature* 2001; **410**: 235-240 [PMID: 11242087 DOI: 10.1038/35065653]

49 **Banerjee R**, Dasgupta A. Specific interaction of hepatitis C virus protease/helicase NS3 with the 3'-terminal sequences of viral positive- and negative-strand RNA. *J Virol* 2001; **75**: 1708-1721 [PMID: 11160669 DOI: 10.1128/jvi.75.4.1708-1721.2001]

50 **DeMarini DJ**, Johnston VK, Konduri M, Gutshall LL, Sarisky RT. Intracellular hepatitis C virus RNA-dependent RNA polymerase activity. *J Virol Methods* 2003; **113**: 65-68 [PMID: 14500128 DOI: 10.1016/S0166-0934(03)00226-X]

51 **Tsuchihara K**, Tanaka T, Hijikata M, Kuge S, Toyoda H, Nomoto A, Yamamoto N, Shimotohno K. Specific interaction of polypyrimidine tract-binding protein with the extreme 3'-terminal structure of the hepatitis C virus genome, the 3'X. *J Virol* 1997; **71**: 6720-6726 [PMID: 9261396]

52 **Ito T**, Tahara SM, Lai MM. The 3'-untranslated region of hepatitis C virus RNA enhances translation from an internal ribosomal entry site. *J Virol* 1998; **72**: 8789-8796 [PMID: 9765423]

53 **McCaffrey AP**, Ohashi K, Meuse L, Shen S, Lancaster AM, Lukavsky PJ, Sarnow P, Kay MA. Determinants of hepatitis C translational initiation in vitro, in cultured cells and mice. *Mol Ther* 2002; **5**: 676-684 [PMID: 12027551 DOI: 10.1006/mthe.2002.0600]

54 **Bradrick SS**, Walters RW, Gromeier M. The hepatitis C virus 3'-untranslated region or a poly(A) tract promote efficient translation subsequent to the initiation phase. *Nucleic Acids Res* 2006; **34**: 1293-1303 [PMID: 16510853 DOI: 10.1093/nar/gkl019]

55 **Song Y**, Friebe P, Tzima E, Jünemann C, Bartenschlager R, Niepmann M. The hepatitis C virus RNA 3'-untranslated region strongly enhances translation directed by the internal ribosome entry site. *J Virol* 2006; **80**: 11579-11588 [PMID: 16971433 DOI: 10.1128/jvi.00675-06]

56 **Bung C**, Bochkaeva Z, Terenin I, Zinovkin R, Shatsky IN, Niepmann M. Influence of the hepatitis C virus 3'-untranslated region on IRES-dependent and cap-dependent translation initiation. *FEBS Lett* 2010; **584**: 837-842 [PMID: 20079737 DOI: 10.1016/j.febslet.2010.01.015]

57 **Ito T**, Lai MM. An internal polypyrimidine-tract-binding protein-binding site in the hepatitis C virus RNA attenuates translation, which is relieved by the 3'-untranslated sequence. *Virology* 1999; **254**: 288-296 [PMID: 9986795 DOI: 10.1006/viro.1998.9541]

58 **Villordo SM**, Gamarnik AV. Genome cyclization as strategy for flavivirus RNA replication. *Virus Res* 2009; **139**: 230-239 [PMID: 18703097 DOI: 10.1016/j.virusres.2008.07.016]

59 **Murakami K**, Abe M, Kageyama T, Kamoshita N, Nomoto A. Down-regulation of translation driven by hepatitis C virus internal ribosomal entry site by the 3' untranslated region of RNA. *Arch Virol* 2001; **146**: 729-741 [PMID: 11402859 DOI: 10.1007/s007050170142]

60   **Imbert I**, Dimitrova M, Kien F, Kieny MP, Schuster C. Hepatitis C virus IRES efficiency is unaffected by the genomic RNA 3'NTR even in the presence of viral structural or non-structural proteins. *J Gen Virol* 2003; **84**: 1549-1557 [PMID: 12771425 DOI: 10.1099/vir.0.18907-0]

61   **Wang H**, Shen XT, Ye R, Lan SY, Xiang L, Yuan ZH. Roles of the polypyrimidine tract and 3' noncoding region of hepatitis C virus RNA in the internal ribosome entry site-mediated translation. *Arch Virol* 2005; **150**: 1085-1099 [PMID: 15747050 DOI: 10.1007/s00705-005-0491-3]

62   **Wood J**, Frederickson RM, Fields S, Patel AH. Hepatitis C virus 3' X region interacts with human ribosomal proteins. *J Virol* 2001; **75**: 1348-1358 [PMID: 11152508 DOI: 10.1128/jvi.75.3.1348-1358.2001]

63   **Tanaka T**, Sugiyama K, Ikeda M, Naganuma A, Nozaki A, Saito M, Shimotohno K, Kato N. Hepatitis C virus NS5B RNA replicase specifically binds ribosomes. *Microbiol Immunol* 2000; **44**: 543-550 [PMID: 10941940 DOI: 10.1111/j.1348-0421.2000.tb02532.x]

Basic Study

Herb-partitioned moxibustion alleviates colon injuries in ulcerative colitis rats

Dan Zhang, Yan-Bo Ren, Kai Wei, Jue Hong, Yan-Ting Yang, Li-Jie Wu, Ji Zhang, Zheng Shi, Huan-Gan Wu, Xiao-Peng Ma

Dan Zhang, Jue Hong, Zheng Shi, Huan-Gan Wu, Xiao-Peng Ma, Laboratory of Acupuncture-moxibustion and Immunology, Shanghai Research Institute of Acupuncture and Meridian, Shanghai University of Traditional Chinese Medicine, Shanghai 200030, China

Yan-Bo Ren, Department of Integrated Traditional Chinese Medicine and Western Medicine, North Branch of Huashan Hospital, Fudan University, Shanghai 201907, China

Kai Wei, Key Laboratory of Systems Biomedicine (Ministry of Education), Shanghai Center for Systems Biomedicine, Shanghai Jiao Tong University, Shanghai 200240, China

Yan-Ting Yang, Li-Jie Wu, Ji Zhang, Xiao-Peng Ma, Yueyang Clinical Medicine School, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

ORCID number: Dan Zhang (0000-0003-0747-8691); Yan-Bo Ren (0000-0003-0738-5591); Kai Wei (0000-0003-1102-1117); Jue Hong (0000-0002-9857-818X); Yan-Ting Yang (0000-0003-1388-4047); Li-Jie Wu (0000-0003-3349-0918); Ji Zhang (0000-0002-6016-1063); Zheng Shi (0000-0003-0726-954X); Huan-Gan Wu (0000-0003-0563-1560); Xiao-Peng Ma (0000-0002-0582-3562).

Author contributions: Zhang D, Ren YB, Wei K and Hong J contributed equally to this work; Ma XP designed and supervised this research; Zhang D and Ren YB performed the animal experiment; Ren YB, Wei K, Hong J, Yang YT and Zhang J contributed to detection of all indexes; Zhang D wrote this manuscript and Wu LJ analyzed the data; Zhang D, Ren YB, Wei K and Hong J revised this manuscript; Shi Z and Wu HG were devoted to the guidance; all authors approved the final version of this article, including the author list.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine.

Conflict-of-interest statement: The authors declare that there is no conflict of interest regarding the publication of this paper.

Data sharing statement: No additional data are available.

The ARRIVE guidelines statement: The ARRIVE Guidelines have been adopted.

Manuscript source: Unsolicited manuscript

Correspondence to: Xiao-Peng Ma, PhD, Professor, Laboratory of Acupuncture-moxibustion and Immunology, Shanghai Research Institute of Acupuncture and Meridian, Shanghai University of Traditional Chinese Medicine, No. 650 South Wanping Road, Xuhui District, Shanghai 200030, China. pengpengma@163.com
Telephone: +86-21-64690257
Fax: +86-21-64382181

Abstract

AIM

To observe the effect of herb-partitioned moxibustion (HPM) on expression of colonic cytokines in ulcerative colitis (UC) rats.

METHODS

A UC rat model was established by protein immunization in combination with topical chemical stimulation. Rats in the HPM group (n = 8) received HPM at bilateral Tianshu (ST25) points. The gross injury and pathological scores of the colon were recorded. The expression profile of colonic cytokines was assayed using the protein microarray technique. Specific differential cytokines were selected and verified by ELISA. The corresponding UniProt Accessions of the differentially expressed cytokines were retrieved in the UniProt database. The pathways involved were analyzed with the help of the KEGG PATHWAY database. The DAVID database was used for functional cluster and pathway analysis.

RESULTS

HPM improved colon injuries in UC rats, manifested by accelerated repair of ulcers and alleviation of inflammation, and the gross injury and pathological scores both significantly decreased ($P < 0.01$). Fold change > 1.3 or < 0.77 was taken as the screening standard. There were 77 down-regulated and 9 up-regulated differentially expressed colonic cytokines in the HPM group compared with the model group, and expression of 20 differed significantly ($P < 0.05$). Twelve of the 20 significantly differentially expressed cytokines [β-catenin, interleukin-1 receptor 6 (IL-1R6), IL-1β, B7-1, nerve growth factor receptor, AMP-activated protein kinase-α1, neuropilin-2, orexin A, adipocyte differentiation-related protein, IL-2, Fas and FasL] were up-regulated in the model group (n = 3, compared with the normal group) but down-regulated in the HPM group (n = 3, compared with the model group). Functional cluster analysis showed that the differentially expressed colonic cytokines in the HPM group regulated apoptosis and protein phosphorylation. KEGG pathway analysis showed that 52 down-regulated and 7 up-regulated differentially expressed colonic cytokines in the HPM group had pathways. The pathways that interacted between the cytokines and their receptors accounted for the largest proportion (28 of the down-regulated and 5 of the up-regulated cytokines).

CONCLUSION

HPM promotes the repair of colon injuries in UC rats, which is related to the regulation of several abnormally expressed cytokines.

Key words: Cytokine expression profile; Ulcerative colitis; Protein microarray; Rats; Herb-partitioned moxibustion

Core tip: Herb-partitioned moxibustion (HPM) has been shown to be effective in treating ulcerative colitis (UC) in recent years as a non-drug external therapy. In this study, we observed its effect on the expression profile of cytokines in UC rat colon. By protein functional cluster analysis and KEGG pathway analysis, we can conclude that the effect of HPM in promoting the repair of colon injuries of UC rats is plausibly related to the regulation of multiple abnormally-expressed cytokines, and the regulation of the signal pathways interacting between the cytokines and their receptors may be its significant immunological mechanism.

INTRODUCTION

As two major types of inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease (CD) are both mainly characterized by chronic nonspecific inflammation of the colon. The incidence of UC is higher than that of CD, and it is increasing annually, with an incidence of 24.3/100000 in Europe and America, and 2.22/100000 in Guangzhou, which is the highest in China[1-3]. As a commonly encountered digestive disorder, UC is often recurrent and persistent, manifested by abdominal pain, diarrhea and bloody or purulent stools, and has an adverse effect on quality of life. Long-term medical treatment may also incur a high cost[4-8]. Immune dysfunction in the intestinal lining is recognized as the biological mechanism in the development of UC. In patients with UC, a large number of immunocytes (T cells, B cells, macrophages and dendritic cells) and cytokines [proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-6, IL-12, IL-17, IL-21, IL-23, and integrin; anti-inflammatory cytokines such as IL-10, transforming growth factor (TGF)-β and IL-35] are abnormally expressed in the colon[9]. The imbalance of cytokines modulated by activated immunocytes should be the initial factor that causes diffuse superficial inflammatory injuries in UC[10,11]. Regulation of the activity of immune cells and expression of cytokines is beneficial to healing the colonic lining and alleviation of inflammation in UC patients[12,13].

During recent years, acupuncture-moxibustion at Qihai (CV6) and bilateral Tianshu (ST25) has been shown to significantly improve symptoms, quality of life and immune homeostasis in the colon of UC patients[14-16]. Acupuncture-moxibustion is characterized by moderate therapeutic effect, content treatment perception and rapid action, and is especially effective in mitigating abdominal pain and diarrhea in UC patients[17]. Compared with oral medications, acupuncture-moxibustion, as a
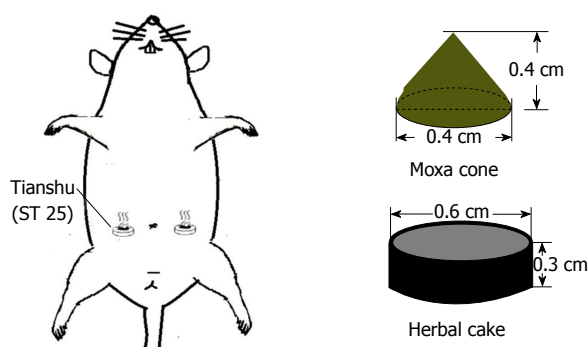
Figure 1 Illustration of herb-partitioned moxibustion. An herbal cake was placed on Tianshu (ST25) point with a moxa cone on the top to ignite. This point is located at the lower 1/3 between the xiphoid process and the midpoint of the pubic symphysis, 0.5 cm away.

nondrug external therapy, can also promote colonic blood circulation and intestinal peristalsis, heal damaged colonic lining, regulate colonic immune reactions, enhance self-healing and reduce relapse[18]. Although the efficacy of acupuncture-moxibustion in treating UC in both the short- and long-term is well recognized, their mechanism of action is still vague. Therefore, it is important to discover how acupuncture-moxibustion works in treating UC, and this will boost its application in the treatment of UC.

Protein microarray is an important tool in profiling the protein expression in IBD and screening new drugs. This technique is known for its high throughput, high sensitivity and high precision, and thoroughly and quantitatively analyzes the genre, number and correlation of proteins, by which complicated network and possible regulatory mechanisms are investigated[19-21]. Protein microarray has been gradually applied to research into traditional Chinese medicine (TCM), such as pharmacodynamics and toxicology of Chinese medications[22-24], yet it has rarely been used in the study of acupuncture-moxibustion therapy.

The present study established experimental UC rats and adopted the protein microarray technique to investigate the effect of herb-partitioned moxibustion (HPM) on the expression profile of colonic cytokines, to select those most associated with the therapeutic action of HPM. We used the UniProt and KEGG PATHWAY databases, as well as DAVID pathway and functional cluster analysis to study the immunopathogenesis of experimental UC and its treatment with HPM from the angle of cytokine expression profile.

MATERIALS AND METHODS

Experimental animals
Twenty-seven male Sprague-Dawley rats weighing 160 ± 20 g were provided by the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine (SCXK (Hu) 2007-0005). The rats were housed for 3 d under controlled conditions (22 ± 2 ℃, relative humidity 64%) before the experiment

started. All procedures for animal experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals recommended by the World Health Organization and were approved by the Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine. All rats were randomly divided into three groups as normal group, model group and HPM group, with nine rats per group.

Reagents and equipment
The following reagents and equipment were used: Freund's adjuvant (Sigma, St Louis, MO, United States), refined moxa wool (Nanyang Hanyi Moxa Co. Ltd., He'nan, China), Fugui formula 1 (Huaji Pharmaceutical Co. Ltd., Shanghai, China), hematoxylin and eosin (HE) staining kit (Nanjing Jiancheng Technology Co. Ltd., Nanjing, China), self-made cone-like copper moxa-cone mold, self-made columnar copper herbal cake mold, biotin label-based rat antibody array (90 rat proteins, RayBiotech, Norcross, GA, United States), ELISA kits for rat Fas/TNFRSF6, FasL/TNFSF6, IL-1R6 (IL-1Rrp2) and IL-1β (Bio-Techne, Minneapolis, MN, United States), phosphatase/protease inhibitor cocktail (Roche, Basel, Switzerland), RIPA lysis buffer, phenylmethanesulfonyl fluoride (PMSF) (Beyotime, Jiangsu, China), microplate reader (Thermo Fisher Scientific, Waltham, MA, United States), scanner (Qinghua Ziguang, Beijing, China), ScanAlyze image analysis software (Stanford University, CA, United States), pathological analysis system (Leica, Wetzlar, Germany), and light microscope and imaging system (Olympus, Tokyo, Japan).

Rat model of UC
The rat model of UC was established using immunization plus topical stimulation with formalin[25,26]. After modeling, each group contributed one rat for histopathological observation to verify the success of the UC model.

HPM
Rats in the HPM group were treated with HPM at bilateral Tianshu (ST25) points (Figure 1) by using a rat fixator. An herbal cake was placed on each point with a moxa cone on the top. The moxa cone was ignited, with two cones for each point as one session. The intervention was conducted once every other day, for four sessions and 8 d in total. Rats in the model group did not receive any interventions except the same grasping and fixing as in the HPM group. Rats in the normal group did not receive any modeling operation or interventions except for the same grasping and fixing.

Each moxa cone was made of 90 mg refined moxa wool by a copper mold, 0.4 cm in diameter and 0.4 cm in height. Guifu formula 1 was used to make herbal cakes, consisting of *Radix aconiti lateralis preparata, Cortex cinnamon, Radix salvia miltiorrhizae, Flos carthami and Radix aucklandiae* (ratio: 10:2:3:3:1). The herbal powder was mixed with yellow rice wine immediately

before HPM treatment and then made into cakes of the same size using a specific mold (0.6 cm in diameter and 0.3 cm thickness).

Colon sample preparation

After the treatment, the rats were euthanized by an intraperitoneal injection of pentobarbital sodium (150 mg/kg). Colons (6-8 cm, cut at 2 cm away from the anus) were collected and opened longitudinally to observe and score gross injuries under a microscope. The scoring standard is shown in Supplementary Table 1. The colons were then cut into 2 pieces. The proximal part was stored at -80 ℃ and the distal part was fixed in 10% neutral-buffered formalin.

Morphological observation of the colon

After fixation in 10% neutral-buffered formalin overnight, colons were dehydrated, embedded in paraffin, sliced (3-5 μm) and baked (60 ℃) using the Leica pathological analysis system, followed by dewaxing and dehydration by dimethylbenzene and graded ethanol. The specimens were stained with hematoxylin for 10 min, differentiated by 1% HCl and ethanol, stained blue by 1% ammonia solution, stained by 0.5% eosin for 3 min, dehydrated through 70%, 85%, 95% and 100% ethanol, made transparent by dimethylbenzene, and sealed in neutral resin. Finally, the colon tissues were observed under a light microscope and scored. The scoring standard is shown in Supplementary Table 2.

Total protein extraction in the colon

Three colon samples were selected from each of the three groups to extract total protein. Lysis buffer containing protease inhibitor was added at 1 mL/250 mg (1 mL RIPA was mixed with 5 μL protease inhibitor solution, 5 μL PMSF and 5 μL phosphatase/protease inhibitor cocktail), and homogenized at a low speed (3000 rpm) for complete lysis of colon tissues. The colonic lysate was centrifuged at 14000 rpm for 15 min. The supernatant was collected to determine the concentration of total protein by bicinchoninic acid method.

Cytokine detection in colon

The protein chips were put into the reaction chamber of the chemiluminescent protein microarray kit from RayBiotech. A total of 2 mL blocking buffer was added into the reaction chamber, interacting for 30 min at room temperature. The protein chips were incubated with 1 mL sample supernatant (content of total proteins 50-500 μg), streptavidin antibody and horseradish-peroxidase-labeled anti-streptavidin antibody in sequence for 2 h at each step. After thorough washing, 500 μL chemiluminescent solution was added to the reaction chamber, which was incubated for 2 min at room temperature. The solution was then removed, and the protein chips were taken for imaging. Ninety kinds of cytokines are detailed in Supplementary Table 3.

Image and data analysis

The images were scanned and saved in .tiff format.

ScanAlyze was used to transform the images into data that were then input into Microsoft Excel for normalization and analysis. Normalized result = [Original value - Blank (average)]/Positive (average). First, the protein abundance of rat colonic cytokines in each group was described. Second, intergroup fold change in normalized protein abundance was calculated. Fold change > 1.3 or < 0.77 were taken as the standard to define upregulation and downregulation of a cytokine, respectively.

UniProt accession and KEGG pathway analysis

Accessions of the differentially expressed cytokines were retrieved in the UniProt database to understand their protein structure and physicochemical properties. The corresponding pathways of the cytokines were sought in the KEGG PATHWAY database and then underwent functional cluster and pathway analysis with DAVID.

Validation of differentially expressed proteins in the colon

ELISA was used to verify the specific differentially expressed colonic cytokines. The appropriate amount of diluent samples, standard products and 10 μL avidin and 50 μL enzyme-labeled reagent were added to a 96-well dish and incubated at 37 ℃ for 30 min. When the above solutions were washed off, 50 μL chromogenic reagents A and B were added successively and mixed. The dish was kept at 37 ℃ for 15 min in the dark to allow developing. Finally, 50 μL stop solution was added to terminate the reaction. A microplate reader from Thermo Fisher was used to determine OD_{450}. The protein concentration was positively correlated with the OD value. The standard curve was drawn to reflect the cytokine concentration.

Statistical analysis

SPSS version 18.0 (IBM, Armonk, NY, United States) was used for statistical analysis. Measurement data including body weight, gross colon injury score, histopathological score, and expression of the 90 cytokines and differentially expressed cytokines, which completely or substantially conformed to a normal distribution and homogeneity of variance, were expressed as means ± SD. Body weight, gross colon injury score, histopathological score and expression of colonic differentially expressed cytokines were analyzed by one-way ANOVA followed by least significant difference test. An independent *t*-test was used for the between-group comparison of the expression of the 90 cytokines. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

General condition

Rats in the normal group were vigorous and had normal intake of food, defecation, ruddy anus and steady increase of body weight. On the contrary, rats in the model group were weak and irritable, with reduced food intake, soft or loose stool sometimes with blood or pus, stained anus, and slower increase of body weight ($P < 0.01$). Compared with the model group, rats in the HPM
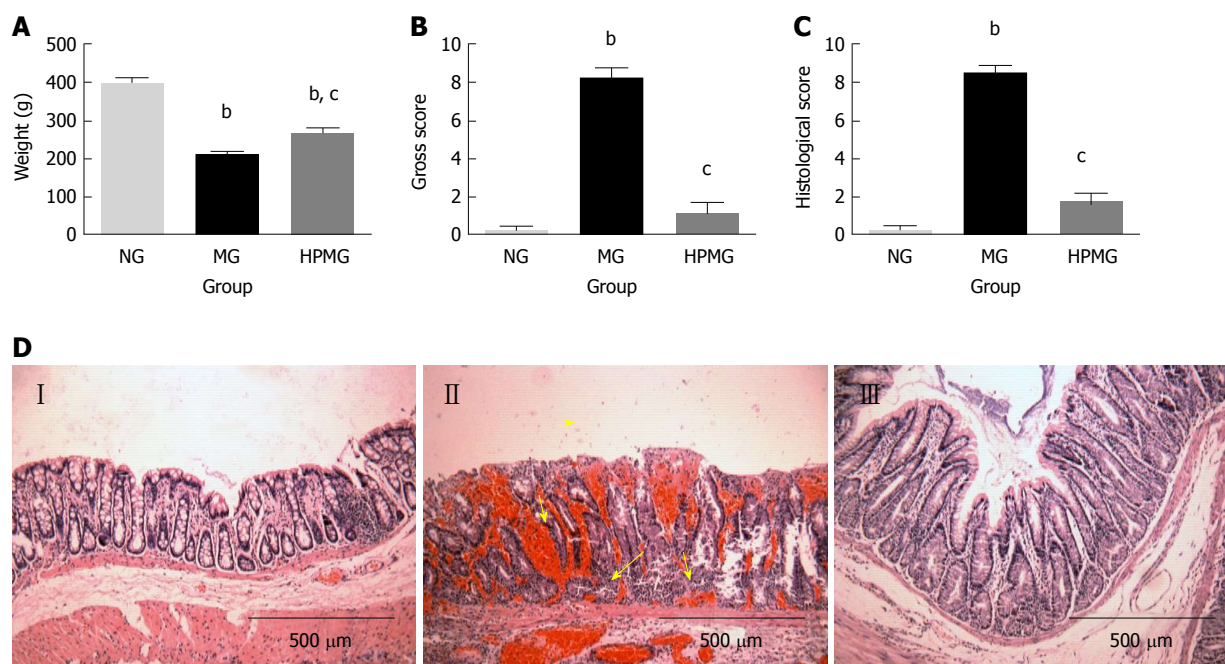
Figure 2 General condition, morphological observation and injury score of colon tissues in each group. A: Weight change of rats in each group; B: Gross injury score of rat colon in each group; C: Histological score of rat colon in each group; D: Histological and morphological structure under microscope by hematoxylin-eosin staining (Amplification × 100). NG (Ⅰ): Normal group, MG (Ⅱ): Model group; HPMG (Ⅲ): Herb-partitioned moxibustion group. Data are presented as the mean ± SD, *n* = 8 per group. [b]*P* < 0.01 (*vs* normal group); [c]*P* < 0.01 (*vs* model group).

group showed improvements in dieting, stool pattern and spiritual state, and body weight increased more significantly (*P* < 0.01) (Figure 2A).

Gross colon injury score

Compared with the normal group, colons in the model group were harder and darker, with thickened intestinal walls, less smooth lining with significant congestion and scattered ulcers covered by discharge, and the gross injury score was significantly higher (*P* < 0.01). Compared with the model group, colons in the HPM group showed notable improvement in colon injuries, presenting with soft pink intestines, slightly thickened intestinal walls, smooth and complete lining without visible ulcers, and the gross injury score was significantly lower (*P* < 0.01) (Figure 2B).

Morphological changes in the colon

Compared with the normal group, the model group had a significantly higher histopathological score for colon injuries (*P* < 0.01) and presented with disconnected colon lining, colonic gland necrosis or missing, disorganized crypts, atrophy in some colonic glands, and infiltration of the lamina propria and submucosa by many inflammatory cells such as neutrophils, eosinophils and mononuclear cells. Compared with the model group, rats in the HPM group showed significant improvements in colon injuries, manifested by substantially complete lining without noticeable ulcers but obvious growth of well-structured colonic glands, coupled with inflammatory polyps, alleviated inflammation in the lamina propria and submucosa, and a significantly lower histopathological score (*P* < 0.01), (Figure 2C and D).

Analysis of cytokine expression profiles

Compared with the normal group, 30 cytokines in colon tissues (*n* = 3) showed > 1.3 fold change in the model group (Table 1 and Figure 3A), among which 22 cytokines were significantly differentially expressed (*P* < 0.05); 34 cytokines showed < 0.77 fold change (Table 2, Figure 3A, and Figure 4), and 4 of them were significantly differentially expressed (*P* < 0.05).

Compared with the model group, 77 colonic cytokines showed < 0.77 fold change in the HPM group (Table 3, Figure 3B, and Figure 4), and 14 of them were significantly differentially expressed (*P* < 0.05); 9 cytokines showed > 1.3 fold change (Table 4 and Figure 3B), and 6 of them were significantly differentially expressed (*P* < 0.05).

Further analysis discovered that 12 cytokines that were upregulated (> 1.3 fold change) in the model group compared with those in the normal group were downregulated (< 0.77 fold change) after HPM intervention (Figure 5): *e.g.*, β-catenin, IL-1 receptor 6 (IL-1R6), B7-1, IL-1β, nerve growth factor receptor, AMP-activated protein kinase-α1, neuropilin-2, orexin A, adipocyte differentiation-related protein, IL-2, Fas and FasL. However, no cytokines that were downregulated (< 0.77 fold change) in the model group compared with those in the normal group were upregulated (> 1.3 fold change) after the intervention with HPM.

Functional cluster of the differentially expressed cytokines

Compared with the normal group, the differentially expressed cytokines in the model group regulated apoptosis, protein phosphorylation and modification,

Table 1 Cytokines associated with the development of ulcerative colitis (up-regulated)

Cytokine (up)	FC (M/N)	*P* value	Cytokine (up)	FC (M/N)	*P* value
FGF-BP	1.362082	0.02	FADD	2.012878	0.15
IL-5	1.383255	0.12	B7-1	2.121888	< 0.00
MIG-6	1.502947	0.01	Fas	2.133077	0.03
IL-10	1.538953	0.04	E-Selectin	2.193775	0.35
Fractalkine	1.562316	0.01	SPP1	2.286921	0.02
PDGF-AA	1.621171	0.05	IL-2	2.298734	0.01
β-Catenin	1.63583	< 0.000	Activin A	2.328823	0.01
FSL1	1.645565	0.004	IL-3	2.356267	0.08
AMPKα1	1.747397	0.001	ADFP	2.429592	< 0.00
RAGE	1.778351	0.02	Fas Ligand	2.476364	0.02
BDNF	1.800268	< 0.00	Neuropilin-2	2.742889	0.02
IC-1	1.812053	0.39	NGFR	3.519845	< 0.00
Insulin	1.917783	0.03	IL-1Rrp2/IL-1R6	3.668512	< 0.00
ACTH	1.919645	0.15	Orexin A	4.768017	0.01
PK1	1.956145	0.08	IL-1β	5.017418	< 0.000

FC: Fold change; M/N: Average of normalized result in the model group/average of normalized result in the normal group.

Figure 3 Protein abundances of colonic cytokines in each group. A: MG *vs* NG in comparing the change in mean protein abundance, and the cytokines with an obvious change are shown in the figure; B: HPMG *vs* MG in comparing the change in mean protein abundance, and the cytokines with an obvious change are shown in the figure. The significantly down-regulated proteins are marked in red (the focus of this study). NG: Normal group; MG: Model group; HPMG: Herb-partitioned moxibustion group.

Table 2 Cytokines associated with the development of ulcerative colitis (down-regulated)

Cytokine (down)	FC (M/N)	*P* value	Cytokine (down)	FC (M/N)	*P* value
MuSK	0.025485	0.26	GM-CSF	0.558641	0.01
MMP-13	0.191098	0.50	TRAIL	0.561294	0.73
ICK	0.206558	0.99	GH	0.583295	0.11
IFN-γ	0.210272	0.60	TLR4	0.605775	0.09
TNF-α	0.232403	0.54	CCR4	0.614899	0.15
IL-1α	0.283395	0.64	VEGF	0.630412	0.62
MIP-3α	0.297499	0.82	TIMP-2	0.636711	0.48
EGFR	0.362763	0.82	TGF-β1	0.644985	0.19
MMP-2	0.366125	0.94	CD106	0.6635	0.04
MIF	0.441963	0.52	ICAM-1	0.701176	0.3
MIP-1α	0.446228	0.69	Thrombospondin	0.708378	0.09
Ubiquitin	0.485159	0.59	IDE	0.711151	0.27
TIMP-3	0.489656	0.65	β-NGF	0.727731	0.13
MIP-2	0.513305	0.68	IC-3	0.748519	0.66
TIE-2	0.528072	0.81	MDC	0.749424	0.13
TGF-β3	0.550782	0.08	VEGF-C	0.768934	0.42
Growth hormone R	0.553239	0.50	CNTF	0.769193	0.02

FC: Fold change; M/N: Average of normalized result in the model group/average of normalized result in the normal group.

proliferation of white blood cells, lymphocytes and mononuclear cells, migration of white blood cells and vascular endothelial cells, activation of chemokines, and modulation of transcription factors of nuclear factor-κB and angiogenesis (Supplementary Tables 4 and 5). Compared with the model group, the differentially expressed cytokines in the HPM group regulated apoptosis, protein phosphorylation, cell migration, protein metabolism, activation and chemotaxis of neutrophils, activation of lymphocytes and transcription factors, migration of vascular endothelial cells, and secretion of cytokines (Supplementary Tables 6 and 7).

KEGG pathway analysis
Compared with the normal group, 18 of the up-regulated differentially expressed cytokines in the model group involved 18 signaling pathways. Eleven of them were pathways that interacted between cytokines and their receptors. The rest were pathways mainly involved in allotransplantation reactions, autoimmune thyroid disorders, type 1 diabetes, network of intestinal immunoglobulins, apoptosis, mitogen-activated protein kinase (MAPK) signaling pathway, graft *vs* host reaction, Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway and Toll-like receptor (TLR) signaling pathway (Figure 6). Twenty-seven of the down-regulated differentially expressed cytokines in the model group involved 21 signaling pathways. Thirteen of them were pathways for the interaction between cytokines and their receptors. The rest were pathways mainly involved in the MAPK signaling pathway, TGF-β signaling pathway, natural killer (NK)-cell-mediated cytotoxicity, JAK/STAT signaling pathway, and apoptosis (Figure 6).

Compared with the model group, 52 of the 77 down-regulated differentially expressed cytokines in the HPM group were involved in 28 signaling pathways. The rest were involved in the JAK/STAT signaling pathway, cancer pathways, network of immunoglobulin A, MAPK

signaling pathway, immunological rejection, TLR signaling pathway, chemokine signaling pathway, apoptosis, intercellular adhesion, graft *vs* host reaction, FcεRI and TGF-β signaling pathways, T-cell receptor pathways, mammalian target of rapamycin signaling pathway, NOD-like receptors and NK-cell-mediated cytotoxicity (Figure 7). There were ten pathways associated with the up-regulated cytokines that were differentially expressed in the HPM group compared with the model group (seven of the nine differentially expressed cytokines involved ten pathways). Five of them were for interactions between cytokines and receptors. The rest were mainly involved in the MAPK signaling pathway, graft *vs* host reaction, transplantation rejection, apoptosis, and TGF-β and TLR signaling pathways (Figure 7).

Verification of specific differential cytokines IL-1β, IL-1R6, Fas and FasL
Based on the protein microarray and functional cluster and pathway analysis, specific differential cytokines IL-1β, IL-1R6, Fas and FasL were selected for further verification by ELISA. Compared with the normal group, there were significant increases in expression of IL-1β, IL-1R6, Fas and FasL in the colon tissues of model group rats ($P < 0.01$, Figure 8). Compared with the model group, expression of IL-1β, IL-1R6, Fas and FasL decreased significantly in the HPM group ($P < 0.01$, Figure 8).

DISCUSSION
Although the development and application of oral medications or biological agents for UC have progressed, the treatment results have differed individually, not to mention that adverse effects have often been reported. Some research has proposed the use of more than two drugs together as a multitarget treatment to boost the efficacy, but more research is needed[27]. Conventional acupuncture-moxibustion therapy can produce a multi-
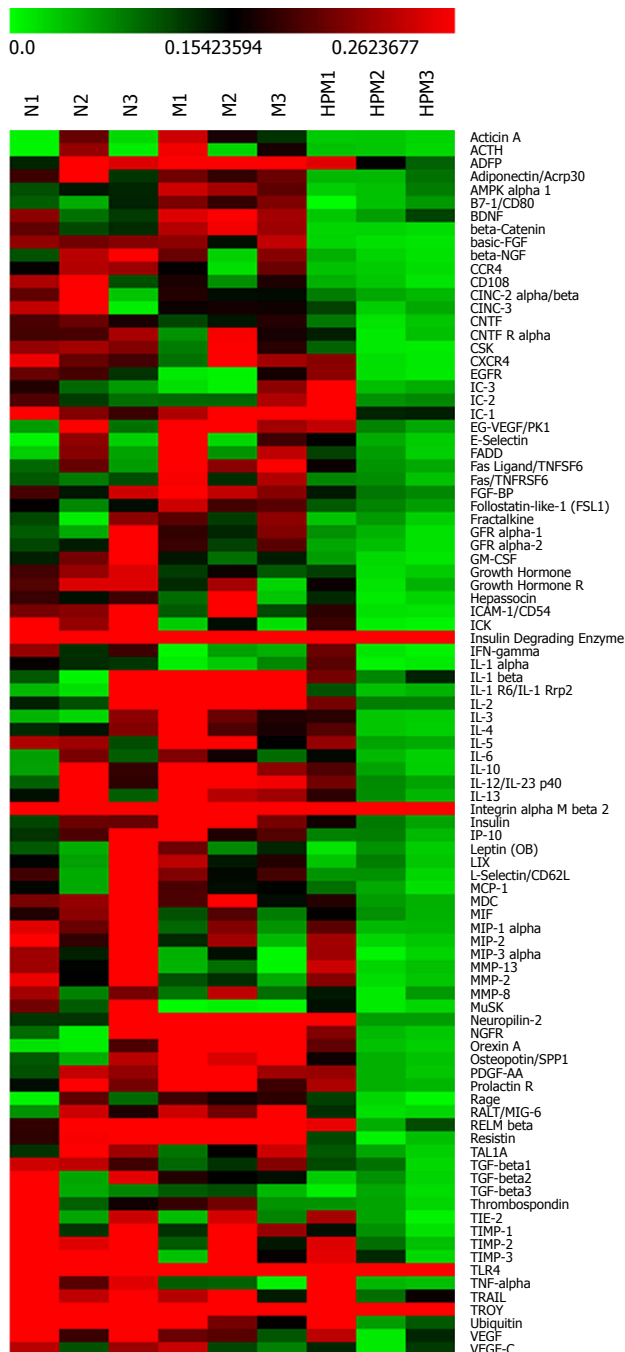
Figure 4 Clustering heatmap of colonic cytokines in each group. Red stands for those higher than the average density (marked in dark); green for those lower than the average density. *n* = 3 rats per group. N: Normal group; M: Model group; HPM: Herb-partitioned moxibustion group.

mostly regulated apoptosis, protein phosphorylation, lymphocyte activation and chemotaxis, protein metabolism, and activation of transcription factors. This indicates that the effect of HPM in promoting the repair of colon injuries in UC rats is possibly linked to the effective regulation of several abnormal cytokines. According to KEGG pathway analysis, 38 pathways were related to these differentially expressed cytokines. The high number suggests a plausible relationship between the immunological action of HPM and the regulation of cytokines, their receptors and the involved pathways in treating UC.

Despite the unclear picture of the pathogenesis of UC, it is widely accepted that chronic nonspecific inflammation in the colon is its cardinal feature. A large number of immunocompetent cells presenting with excessive chemotaxis and abnormally expressed cytokines have been found in the colon of UC patients. Cytokines and their receptors, as well as the factors regulating signaling pathways, mainly serve to regulate the molecular network of colonic epithelial cells, immunocytes in lamina propria, and bacteria. Therefore, abnormal cytokines are possibly a crucial factor in causing immune disorders and colon injuries in UC[28,29]. Cytokines form a complicated network, interacting with immunocytes both as cause and effect, but their mechanism of action is still unclear. According to previous studies, the cytokines related to UC are comparatively precise, mainly focused on inflammatory factors, anti-inflammatory factors, chemokines and lymphocyte-activating factors, with a limited quantity for measurement. The present study has made up for the previous studies in genre and quantity of cytokines by examining important factors involved in apoptosis, cell proliferation, signaling pathway regulation, protein modification, tumor, and intestinal immune network. Besides the focus on inflammation, this study also used UniProt and KEGG pathway databases to further explore the possible pathogenesis of UC, providing new insights and foundations for UC and clinical intervention studies in the future.

HPM integrates moxibustion and Chinese herbal medicine and produces both pharmaceutical effects on the skin and physical thermal effects. It has been shown that HPM can exert definite effects in treating mild-to-moderate UC, by boosting colon healing and relief of inflammation; thus it is well accepted by patients[17]. Compared with oral medication and surgery, HPM is simple to operate, gentle in action, consistent in efficacy and extensive in regulation. It can significantly improve quality of life and restore intestinal function[30-32]. More and more cytokine antibodies have been approved for treatment of IBD, such as anti-TNF (*e.g.*, infliximab, etanercept and adalimumab) and anti-IL-12/23 p40 (*e.g.*, ustekinumab), indicating that regulation of cytokines is practical and essential in treating colitis[33]. How does HPM exert its effect on UC? Is it also related to the regulation of cytokines, and what else is involved? In this study, protein microarrays revealed that 12 cytokines

system, multitarget and multidirectional effect, without causing notable adverse reactions. For all these merits, it is recommended as an effective therapeutic approach for UC. Establishment of the mechanism of action of HPM in treating UC could provide important evidence to promote its application in treating IBD. The present study was a pilot study that used high-throughput protein microarray to elucidate the mechanism of action of HPM in the treatment of UC. Here, 86 differentially expressed cytokines in the colon were triggered by HPM. They

Table 3 Cytokines associated with the action of herb-partitioned moxibustion (down-regulated)

Cytokine (down)	FC (H/M)	*P* value	Cytokine (down)	FC (H/M)	*P* value
basic-FGF	0.0978	0.70	MCP-1	0.322376	0.71
β-Catenin	0.099918	0.03	PDGF-AA	0.329641	0.13
IL-1Rrp2/IL-1R6	0.157933	0.03	TGF-β3	0.335626	0.50
Resistin	0.165169	0.45	FADD	0.338264	0.33
B7-1/CD80	0.165526	0.02	Prolactin R	0.352073	0.57
Activin A	0.169884	0.23	IL-10	0.353484	0.23
IL-1β	0.181118	0.01	IL-3	0.354765	0.15
NGFR	0.205767	0.02	FSL1	0.374421	0.06
β-NGF	0.206263	0.52	PK1	0.374728	0.22
ACTH	0.206715	0.50	CINC-2 α/β	0.375162	0.76
AMPKα1	0.218363	0.01	FGF-BP	0.378172	0.21
CSK	0.220766	0.54	ICAM-1	0.379167	0.45
GFRα-2	0.222922	0.65	TAL1A	0.379464	0.52
CCR4	0.229987	0.26	CNTF Rα	0.380412	0.57
MIG-6	0.234297	0.22	IL-13	0.381048	0.52
Neuropilin-2	0.23433	0.04	IL-12	0.395678	0.55
Fractalkine	0.238117	0.39	CXCR4	0.397749	0.92
CD106	0.238859	0.32	IL-4	0.400025	0.30
GFRα-1	0.248202	0.66	CINC-3	0.404805	0.89
Orexin A	0.256555	0.01	MDC	0.417278	0.50
GM-CSF	0.259198	0.16	IL-5	0.41803	0.33
TGF-β2	0.261203	0.58	E-Selectin	0.424327	0.43
LIX	0.270135	0.91	Hepassocin	0.428314	0.61
BDNF	0.270917	0.06	GH	0.441903	0.02
RAGE	0.27422	0.24	Integrin αMβ2	0.458492	0.03
Leptin (OB)	0.276614	0.79	IL-6	0.47843	0.60
SPP1	0.278682	0.07	TGF-β1	0.511556	0.12
TIMP-1	0.280948	0.88	MMP-8	0.525606	0.65
L-Selectin	0.283725	0.91	GH R	0.581019	0.17
Fas	0.289612	0.06	TIMP-2	0.581976	0.31
RELMβ	0.290633	0.47	VEGF-C	0.610166	0.54
ADFP	0.290674	0.01	IC-1	0.639561	0.12
Acrp30	0.291541	0.81	IDE	0.678174	0.01
IP-10	0.295663	0.88	TIMP-3	0.69365	0.16
IL-2	0.298121	0.03	MIP-2	0.713848	0.13
Insulin	0.305481	0.09	MIF	0.725496	0.18
CNTF	0.310717	0.10	Ubiquitin	0.73656	0.09
Fas Ligand	0.319733	< 0.05	MIP-1α	0.761068	0.09
Thrombospondin	0.322261	0.55	VEGF	0.765926	0.13

FC: Fold change; H/M: Average of normalized result in the herb-partitioned moxibustion group/average of normalized result in the model group.

Table 4 Cytokines associated with the action of herb-partitioned moxibustion (up-regulated)

Cytokine (up)	FC (H/M)	*P* value	Cytokine (up)	FC (H/M)	*P* value
EGFR	1.335106	0.13	TNF-α	1.937065	0.04
TROY	1.34044	0.31	IFN-γ	2.000647	0.01
TLR4	1.511846	0.04	MMP-13	2.19335	0.04
IC-3	1.664604	0.74	MuSK	12.24563	0.02
IL-1α	1.845212	0.01			

FC: Fold change; H/M: Average of normalized result in the herb-partitioned moxibustion group/average of normalized result in the model group.

upregulated (fold change > 1.3) in the model group compared with the normal group were downregulated (fold change < 0.77) after HPM intervention. These cytokines are involved in the modulation of inflammation, apoptosis, adhesion, activation of co-stimulatory signals, metabolism, and nervous regulation. The pathway analysis also suggested an association between the effect of HPM in healing colon injuries of UC and the downregulation of several abnormally expressed cytokines.

Hence, regulation of signaling pathways of cytokines and their receptors is possibly an important immunological mechanism. So far, we have seen that HPM has several regulatory effects on colonic cytokines. However, it remains a question whether this non-drug therapy can compare with oral or venous application of antibodies, immunosuppressive agents, steroidal and anti-inflammatory drugs.

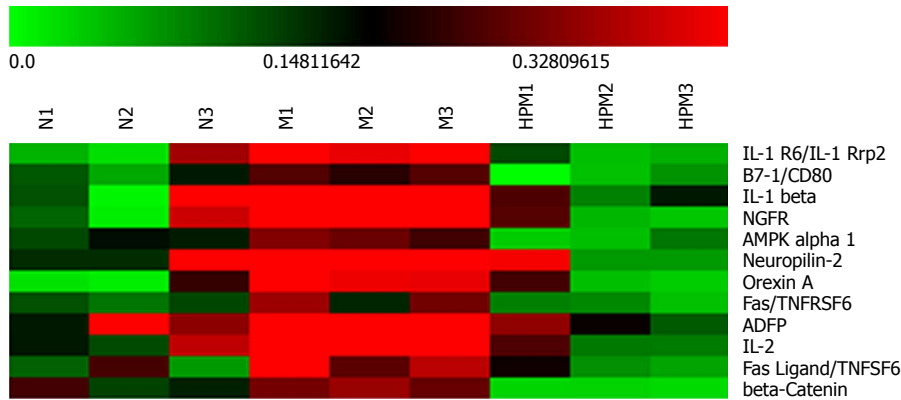We showed that HPM significantly downregulated

Figure 5 Clustering heatmap of the specific differential cytokines in each group. Red stands for those higher than the average density (marked in dark); green for those lower than the average density. *n* = 3 rats per group. N: Normal group; M: Model group; HPM: Herb-partitioned moxibustion group.
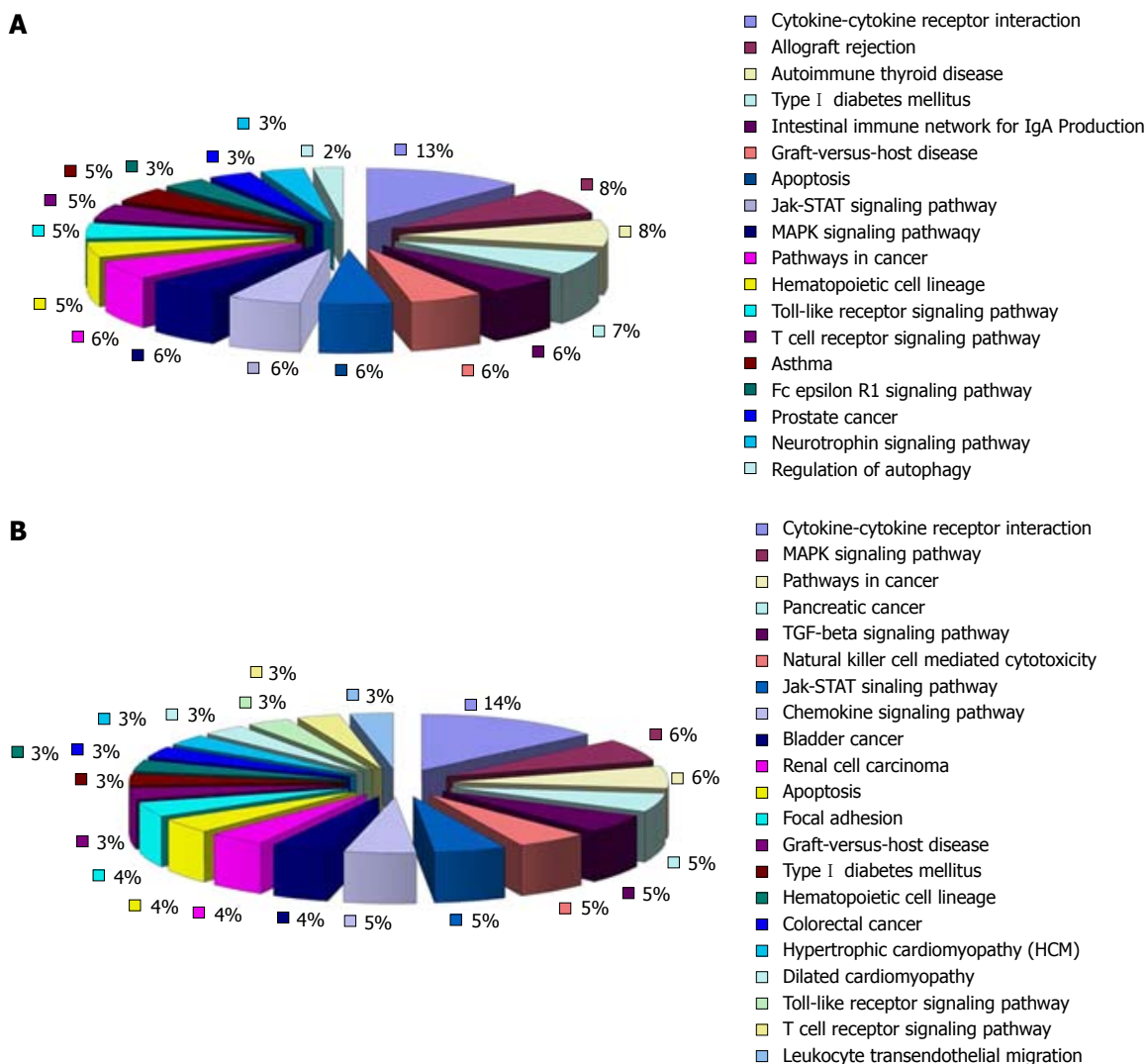

Figure 6 KEGG pathway analysis of the cytokines related to the development of ulcerative colitis. A: Eleven of all the major signaling pathways of the up-regulated cytokines in the model group (compared with the normal group) are involved in the interaction between cytokines and their receptors (36.7%); B: Of the major signaling pathways of the down-regulated cytokines in the model group (compared with the normal group), 13 pathways regulate the interaction between cytokines and their receptors (39.4%) (Supplementary Tables 4 and 5).

the expression of IL-1β, IL-1R6, Fas and FasL, which agreed with previous studies[16,34-40]. We demonstrated

that inflammatory infiltration and apoptosis in the colonic epithelium, controlled by cytokines and their network,

A

- Cytokine-cytokine receptor interaction
- Graft-versus-host disease
- Type I diabetes mellitus
- MAPK signaling pathway
- Allograft rejection
- Hematopoietic cell lineage
- Apoptosis
- TGF-beta signaling pathway
- Systemic lupus erythematosus
- Toll-like receptor signaling pathway

B

- Cytokine-cytokine receptor interaction
- Jak STAT signaling pathway
- Pathways in cancer
- Intestinal immune network for IgA production
- MAPK signaling pathway
- Allograft rejection
- Toll-like receptor signaling pathway
- Chemokine signaling pathway
- Type I diabetes mellitus
- Autoimmune thyroid disease
- Hematopoietic cell lineage
- Graft-versus-host disease
- Renal cell carcinoma
- Apoptosis
- Cell adhesion molecules (CAMs)
- Focal adhesion
- Asthma
- Pancreatic cancer
- Fc epsilon RI signaling pathway
- Hypertrophic cardiomyopathy (HCM)
- TGF-beta signaling pathway
- T cell receptor signaling pathway
- mTOR signaling pathway
- NOD-like receptor signaling pathway
- Colorectal cancer
- Natural killer cell mediated cytotoxicity
- Leukocyte transendothelial migraton
- Cytosolic DNA-sensing pathway

Figure 7 KEGG pathway analysis of the cytokines related to the action of herb-partitioned moxibustion. A: Among the major signaling pathways related to the up-regulated cytokines in the herb-partitioned moxibustion group (compared with the model group), five pathways were involved in the interaction between cytokines and their receptors (55.6%); B: Of the major signaling pathways related to the down-regulated cytokines in the herb-partitioned moxibustion group (compared with the model group), 27 pathways were involved in the interaction between cytokines and their receptors (38%) (Supplementary Tables 6 and 7).

are crucial factors in the pathogenesis of UC. We also discovered that the proinflammatory factor IL-1β was significantly upregulated, while the immunoregulatory factor TGF-β was down-regulated in UC. This caused an imbalance between proinflammatory and anti-inflammatory factors, which also agreed with previous studies. However, in our study, the proinflammatory factors TNF-α and IFN-γ were downregulated, although without significance, which differed from the previous results. The UC modeling method could have accounted for this difference. IL-1β originates from the innate intestinal immune system, while TNF-α and IFN-γ are produced by T helper cells of the acquired immune system. Our study used immunological and chemical stimulation to establish the UC model, which may have a different mechanism of injury from that induced by bacteria[41,42]. By using protein microarray, we systematically discussed the pathogenesis of UC and possible action mechanism of HPM and hope to inspire more studies to further verify the findings.

ARTICLE HIGHLIGHTS

Research background
The imbalance of cytokines modulated by activated immunocytes has been verified as an initial factor inducing inflammatory injuries in ulcerative colitis (UC). Herb-partitioned moxibustion (HPM), a non-drug external therapy, produces valid efficacy in treating UC, but its potential mechanism is still unclear.

Research motivation
The action mechanism of HPM was explored by using high throughout analysis of cytokine expression profiles in the colon and their network effects in our research.

Research objectives
By identifying the key cytokines in the action of HPM and analyzing their signal pathways, our research aimed to provide research ideas and crucial targets for further elaboration of the anti-inflammation mechanism of HPM in treating UC.

Research methods
A UC rat model was established by protein immunization in combination with topical chemical stimulation. Rats in the HPM group received HPM at bilateral Tianshu (ST25) points. The expression profile of colonic cytokines was assayed

Figure 8 Validation of the specific differential cytokines. A: Expression of IL-1β protein in colon; B: Expression of IL-1Rrp2/IL-1R6 protein in colon; C: Expression of Fas protein in colon; D: Expression of FasL protein in colon. Data are presented as the mean ± SD, *n* = 8 rats per group. [b]*P* < 0.01, [a]*P* < 0.05, (*vs* normal group); [c]*P* < 0.01 (*vs* model group). NG: Normal group; MG: Model group; HPMG: Herb-partitioned moxibustion group.

using the protein microarray technique.

Research results

Seventy-seven down-regulated and nine up-regulated differentially-expressed colonic cytokines were found in the HPM group. Functional cluster analysis showed that the differentially-expressed colonic cytokines in the HPM group regulated apoptosis and protein phosphorylation. KEGG pathway analysis showed that the pathways interacting between the cytokines and their receptors accounted for the largest proportion (28 of 52 down-regulated cytokines and 5 of 7 up-regulated cytokines).

Research conclusions

HPM promotes the repair of colon injuries in UC rats, which is related to the regulation of several abnormally-expressed cytokines.

Research perspectives

By functional cluster and KEGG pathway analyses, this study selected specific differential cytokines in HPM treatment of UC, which can provide potential targets for targeted therapy in the future and also research ideas for further elaboration of signal pathways in the action of HPM for UC. The results showed that the signal pathways interacting between cytokines and their receptors were closely related to the action of HPM, and the MAPK signaling pathway and JAK/STAT signaling pathway were possibly involved in the anti-inflammation process of HPM for colitis. A focus on this field may help better understand the mechanism of moxibustion in treating UC.

REFERENCES

1   **Molodecky NA**, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 2012; **142**: 46-54.e42; quiz e30 [PMID: 22001864 DOI: 10.1053/j.gastro.2011.10.001]

2   **Cosnes J**, Gower-Rousseau C, Seksik P, Cortot A. Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology* 2011; **140**: 1785-1794 [PMID: 21530745 DOI: 10.1053/j.gastro.2011.01.055]

3   **Ng WK**, Wong SH, Ng SC. Changing epidemiological trends of inflammatory bowel disease in Asia. *Intest Res* 2016; **14**: 111-119 [PMID: 27175111 DOI: 10.5217/ir.2016.14.2.111]

4   **Włodarczyk M**, Sobolewska-Włodarczyk A, Stec-Michalska K, Fichna J, Wiśniewska-Jarosińska M. The influence of family pattern abnormalities in the early stages of life on the course of inflammatory bowel diseases. *Pharmacol Rep* 2016; **68**: 852-858 [PMID: 27199029 DOI: 10.1016/j.pharep.2016.04.008]

5   **Bernklev T**, Jahnsen J, Schulz T, Sauar J, Lygren I, Henriksen M, Stray N, Kjellevold Ø, Aadland E, Vatn M, Moum B. Course of disease, drug treatment and health-related quality of life in patients with inflammatory bowel disease 5 years after initial diagnosis. *Eur J Gastroenterol Hepatol* 2005; **17**: 1037-1045 [PMID: 16148548 DOI: 10.1097/00042737-200510000-00006]

6   **Zeitz J**, Ak M, Müller-Mottet S, Scharl S, Biedermann L, Fournier N, Frei P, Pittet V, Scharl M, Fried M, Rogler G, Vavricka S; Swiss IBD Cohort Study Group. Pain in IBD Patients: Very Frequent and Frequently Insufficiently Taken into Account. *PLoS One* 2016; **11**: e0156666 [PMID: 27332879 DOI: 10.1371/journal.pone.0156666]

7   **Casellas F**, López-Vivancos J, Casado A, Malagelada JR. Factors affecting health related quality of life of patients with inflammatory bowel disease. *Qual Life Res* 2002; **11**: 775-781 [PMID: 12482161 DOI: 10.1023/A:1020841601110]

8   **Burkhalter H**, Stucki-Thür P, David B, Lorenz S, Biotti B, Rogler G, Pittet V. Assessment of inflammatory bowel disease patient's needs and problems from a nursing perspective. *Digestion* 2015; **91**: 128-141 [PMID: 25677558 DOI: 10.1159/000371654]

9   **Ince MN**, Elliott DE. Immunologic and molecular mechanisms in inflammatory bowel disease. *Surg Clin North Am* 2007; **87**: 681-696 [PMID: 17560420 DOI: 10.1016/j.suc.2007.03.005]

10   **de Souza HS**, Fiocchi C. Immunopathogenesis of IBD: current state of the art. *Nat Rev Gastroenterol Hepatol* 2016; **13**: 13-27 [PMID: 26627550 DOI: 10.1038/nrgastro.2015.186]

11   **Loddo I**, Romano C. Inflammatory Bowel Disease: Genetics, Epigenetics, and Pathogenesis. *Front Immunol* 2015; **6**: 551 [PMID: 26579126 DOI: 10.3389/fimmu.2015.00551]

12   **Fiorino G**, Bonovas S, Cicerone C, Allocca M, Furfaro F, Correale C, Danese S. The safety of biological pharmacotherapy for the treatment of ulcerative colitis. *Expert Opin Drug Saf* 2017; **16**: 437-443 [PMID: 28279079 DOI: 10.1080/14740338.2017.1298743]

13   **Katsanos KH**, Papadakis KA. Inflammatory Bowel Disease: Updates on Molecular Targets for Biologics. *Gut Liver* 2017; **11**: 455-463 [PMID: 28486793 DOI: 10.5009/gnl16308]

14   **Joos S**, Wildau N, Kohnen R, Szecsenyi J, Schuppan D, Willich SN, Hahn EG, Brinkhaus B. Acupuncture and moxibustion in the treatment of ulcerative colitis: a randomized controlled study. *Scand J Gastroenterol* 2006; **41**: 1056-1063 [PMID: 16938719 DOI: 10.1080/00365520600580688]

15   **Wu H**, Chen H, Hua X, Shi Z, Zhang L, Chen J. Clinical therapeutic effect of drug-separated moxibustion on chronic diarrhea and its immunologic mechanisms. *J Tradit Chin Med* 1997; **17**: 253-258 [PMID: 10437206]

16   **Zhou EH**, Liu HR, Wu HG, Shi Z, Zhang W, Zhu Y, Shi DR, Zhou S. Down-regulation of protein and mRNA expression of IL-8 and ICAM-1 in colon tissue of ulcerative colitis patients by partition-herb moxibustion. *Dig Dis Sci* 2009; **54**: 2198-2206 [PMID: 19083096 DOI: 10.1007/s10620-008-0620-4]

17   **Ji J**, Huang Y, Wang XF, Ma Z, Wu HG, Im H, Liu HR, Wu LY, Li J. Review of Clinical Studies of the Treatment of Ulcerative Colitis Using Acupuncture and Moxibustion. *Gastroenterol Res Pract* 2016; **2016**: 9248589 [PMID: 27885326 DOI: 10.1155/2016/9248589]

18   **Ji J**, Lu Y, Liu H, Feng H, Zhang F, Wu L, Cui Y, Wu H. Acupuncture and moxibustion for inflammatory bowel diseases: a systematic review and meta-analysis of randomized controlled trials. *Evid Based Complement Alternat Med* 2013; **2013**: 158352 [PMID: 24204388 DOI: 10.1155/2013/158352]

19   **Mourad FH**, Yau Y, Wasinger VC, Leong RW. Proteomics in Inflammatory Bowel Disease: Approach Using Animal Models. *Dig Dis Sci* 2017; **62**: 2266-2276 [PMID: 28717845 DOI: 10.1007/s10620-017-4673-0]

20   **Chan PP**, Wasinger VC, Leong RW. Current application of proteomics in biomarker discovery for inflammatory bowel disease. *World J Gastrointest Pathophysiol* 2016; **7**: 27-37 [PMID: 26909226 DOI: 10.4291/wjgp.v7.i1.27]

21   **Yau Y**, Leong RW, Zeng M, Wasinger VC. Proteomics and metabolomics in inflammatory bowel disease. *J Gastroenterol Hepatol* 2013; **28**: 1076-1086 [PMID: 23489082 DOI: 10.1111/jgh.12193]

22   **Chen L**, Hou Q, Zhou ZZ, Li MR, Zhong LZ, Deng XD, Zhu ZY, Cheng ZY, Zhu J, Xiang CL, He WJ, Fu XB. Comparative Proteomic Analysis of the Effect of the Four-Herb Chinese Medicine ANBP on Promoting Mouse Skin Wound Healing. *Int J Low Extrem Wounds* 2017; **16**: 154-162 [PMID: 28741388 DOI: 10.1177/1534734617720623]

23   **Teschke R**, Eickhoff A. Herbal hepatotoxicity in traditional and modern medicine: actual key issues and new encouraging steps. *Front Pharmacol* 2015; **6**: 72 [PMID: 25954198 DOI: 10.3389/fphar.2015.00072]

24   **Fang H**, Wang K, Zhang J. Transcriptome and proteome analyses of drug interactions with natural products. *Curr Drug Metab* 2008; **9**: 1038-1048 [PMID: 19075620 DOI: 10.2174/138920008786927802]

25   **Xu SY**, Bian RL, Chen X. Pharmacology Experiment Methodology. Beijing, China: People's Medical Publishing House 2002: 1335

26   **Wang X**, Liu Y, Dong H, Wu L, Feng X, Zhou Z, Zhao C, Liu H, Wu H. Herb-Partitioned Moxibustion Regulates the TLR2/NF-κB Signaling Pathway in a Rat Model of Ulcerative Colitis. *Evid Based Complement Alternat Med* 2015; **2015**: 949065 [PMID: 26339273 DOI: 10.1155/2015/949065]

27   **Di Sario A**, Bendia E, Schiadà L, Sassaroli P, Benedetti A. Biologic Drugs in Crohn's Disease and Ulcerative Colitis: Safety Profile. *Curr Drug Saf* 2016; **11**: 55-61 [PMID: 26882354 DOI: 10.2174/1574886311101160212171757]

28   **Kunkel EJ**, Campbell DJ, Butcher EC. Chemokines in lymphocyte trafficking and intestinal immunity. *Microcirculation* 2003; **10**: 313-323 [PMID: 12851648 DOI: 10.1038/sj.mn.7800196]

29   **Beck PL**, Wallace JL. Cytokines in inflammatory bowel disease. *Mediat of Inflammat* 1997; **6**: 95-103 [PMID: 18472842 DOI: 10.1080/09629359791785]

30   **Li H**, He T, Xu Q, Li Z, Liu Y, Li F, Yang BF, Liu CZ. Acupuncture and regulation of gastrointestinal function. *World J Gastroenterol* 2015; **21**: 8304-8313 [PMID: 26217082 DOI: 10.3748/wjg.v21.i27.8304]

31   **Takahashi T**. Effect and mechanism of acupuncture on gastrointestinal diseases. *Int Rev Neurobiol* 2013; **111**: 273-294 [PMID: 24215928 DOI: 10.1016/B978-0-12-411545-3.00014-6]

32   **Cheifetz AS**, Gianotti R, Luber R, Gibson PR. Complementary and Alternative Medicines Used by Patients With Inflammatory Bowel Diseases. *Gastroenterology* 2017; **152**: 415-429.e15 [PMID: 27743873 DOI: 10.1053/j.gastro.2016.10.004]

33   **Danese S**, Vuitton L, Peyrin-Biroulet L. Biologic agents for IBD: practical insights. *Nat Rev Gastroenterol Hepatol* 2015; **12**: 537-545 [PMID: 26284562 DOI: 10.1038/nrgastro.2015.135]

34   **De Santis S**, Kunde D, Galleggiante V, Liso M, Scandiffio L, Serino G, Pinto A, Campiglia P, Sorrentino R, Cavalcanti E, Santino A, Caruso ML, Eri R, Chieppa M. TNFα deficiency results in increased IL-1β in an early onset of spontaneous murine colitis. *Cell Death Dis* 2017; **8**: e2993 [PMID: 28796256 DOI: 10.1038/cddis.2017.397]

35   **Fonseca-Camarillo G**, Yamamoto-Furusho JK. Immunoregulatory Pathways Involved in Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2015; **21**: 2188-2193 [PMID: 26111210 DOI: 10.1097/MIB.0000000000000477]

36   **Yao J**, Cao X, Zhang R, Li YX, Xu ZL, Zhang DG, Wang LS, Wang JY. Protective Effect of Baicalin Against Experimental Colitis via Suppression of Oxidant Stress and Apoptosis. *Pharmacogn Mag* 2016; **12**: 225-234 [PMID: 27601854 DOI: 10.4103/0973-1296.186342]

37   **Iwamoto M**, Koji T, Makiyama K, Kobayashi N, Nakane PK. Apoptosis of crypt epithelial cells in ulcerative colitis. *J Pathol* 1996; **180**: 152-159 [PMID: 8976873 DOI: 10.1002/(SICI)1096-9896(199610)180:2<152::AID-PATH649>3.0.CO;2-Y]

38   **O'Neill LA**. The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. *Immunol Rev* 2008; **226**: 10-18 [PMID: 19161412 DOI: 10.1111/j.1600-065X.2008.00701.x]

39   **Wu HG**, Liu HR, Tan LY, Gong YJ, Shi Y, Zhao TP, Yi Y, Yang Y. Electroacupuncture and moxibustion promote neutrophil apoptosis and improve ulcerative colitis in rats. *Dig Dis Sci* 2007; **52**: 379-384 [PMID: 17211698 DOI: 10.1007/s10620-006-9561-y]

40   **Ma TM**, Xu N, Ma XD, Bai ZH, Tao X, Yan HC. Moxibustion regulates inflammatory mediators and colonic mucosal barrier in ulcerative colitis rats. *World J Gastroenterol* 2016; **22**: 2566-2575 [PMID: 26937144 DOI: 10.3748/wjg.v22.i8.2566]

41   **Singh UP**, Singh NP, Murphy EA, Price RL, Fayad R, Nagarkatti

M, Nagarkatti PS. Chemokine and cytokine levels in inflammatory bowel disease patients. *Cytokine* 2016; **77**: 44-49 [PMID: 26520877 DOI: 10.1016/j.cyto.2015.10.008]

42   **Guan Q**, Zhang J. Recent Advances: The Imbalance of Cytokines in the Pathogenesis of Inflammatory Bowel Disease. *Mediators Inflamm* 2017; **2017**: 4810258 [PMID: 28420941 DOI: 10.1155/2017/4810258]

Basic Study

Novel sericin-based hepatocyte serum-free medium and sericin's effect on hepatocyte transcriptome

Yun Huang, Qing Peng, Hai-Yan Li, Zhi-Dong Jia, Yang Li, Yi Gao

Yun Huang, Qing Peng, Hai-Yan Li, Zhi-Dong Jia, Yang Li, Department of Hepatobiliary Surgery Ⅱ, Guangdong Provincial Research Center for Artificial Organ and Tissue Engineering, Guangzhou Clinical Research and Transformation Center for Artificial Liver, Institute of Regenerative Medicine, Zhujiang Hospital, Southern Medical University, Guangzhou 510280, Guangdong Province, China

Yi Gao, Department of Hepatobiliary Surgery Ⅱ, Guangdong Provincial Research Center for Artificial Organ and Tissue Engineering, Guangzhou Clinical Research and Transformation Center for Artificial Liver, Institute of Regenerative Medicine, Zhujiang Hospital, Southern Medical University, Guangzhou 510280, Guangdong Province, China

Yi Gao, State Key Laboratory of Organ Failure Research, Southern Medical University, Guangzhou 510515, Guangdong Province, China

ORCID number: Yun Huang (0000-0002-4247-8964); Qing Peng (0000-0002-7790-6455); Hai-Yan Li (0000-0002-3842-8964); Zhi-Dong Jia (0000-0001-5362-5205); Yang Li (0000-0003-4718-1825); Yi Gao (0000-0003-3525-0133).

Author contributions: Huang Y performed the whole process of this study, including the concept, study design, operation of the experiments, data analysis, statistics and article drafting; Peng Q assisted with study design and critical revision of the article; Li HY, Jia ZD and Li Y assisted with the operation of the experiments; Gao Y laid out the concept and performed the critical revision and final approval of the article.

Conflict-of-interest statement: All authors declare no conflicts-of-interest related to this article.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Yi Gao, MD, PhD, Chief Doctor, Professor, Surgeon, Department of Hepatobiliary Surgery Ⅱ, Guangdong Provincial Research Center for Artificial Organ and Tissue Engineering, Guangzhou Clinical Research and Transformation Center for Artificial Liver, Institute of Regenerative Medicine, Zhujiang Hospital of Southern Medical University, 253 Middle Industrial Avenue, Guangzhou 510515, Guangdong Province, China. gaoyi6146@163.com
Telephone: +86-18922345399
Fax: +86-20-61643207

Abstract

AIM
To develop a novel hepatocyte serum-free medium based on sericin, and to explore the effect of sericin on the hepatocyte transcriptome.

METHODS
A controlled trial comparing novel serum-free medium and other media: C3A cells were cultured in our novel serum-free medium, HepatoZYME, complete medium (DMEM/F12 with 100 mL/L FBS), and DMEM/F12, and

then cell attachment, proliferation, and function as well as the biocompatibility of the media were assessed. A comparative study of serum-free media with or without 2 mg/mL sericin: the effect of sericin on C3A growth was assessed by cell viability and proliferation, the effect of sericin on C3A cell cycle distribution was determined by flow cytometry, and the effect of sericin on the C3A transcriptome was assessed by gene-chip array and RT-qPCR.

RESULTS

More C3A cells attached to the plate containing our serum-free medium than to those containing HepatoZYME and DMEM/F12 at 24 h post-seeding. Both the viability and proliferation rate of C3A cells in sericin-based serum-free medium were superior to those of cells in HepatoZYME and DMEM/F12 ($P < 0.001$). The content of albumin and urea in our serum-free medium was significantly higher than that in HepatoZYME and DMEM/F12 throughout the whole culture period ($P < 0.001$) and was similar to that in complete medium at day 3, 4, and 5. In part 2, cell viability and proliferation were greater in the presence of 2 mg/mL sericin ($P < 0.001$), as was the proportion of cells in S phase (16.21% ± 0.98% *vs* 12.61% ± 0.90%, $P < 0.01$). Gene-chip array analysis indicated that the expression of *CCR6*, *EGFR*, and *FOS* were up-regulated by 2 mg/mL sericin, and RT-qPCR revealed that the expression of *CCR6*, *EGFR*, *FOS*, *AKT1*, *JNK1*, *NFkB1*, *MMP-9*, *MEK2*, *ERK1/2* and *MYC* was up-regulated by 2 mg/mL sericin ($P < 0.05$).

CONCLUSION

We developed a novel hepatocyte serum-free medium. Sericin probably enhances cell attachment through the CCR6-Akt-JNK-NF-κB pathway and promotes cell proliferation through CCR6-mediated activation of the ERK1/2-MAPK pathway.

Key words: Sericin; Serum-free medium; MAPK pathway; Bioartificial liver support system; *CCR6*

Core tip: In recent decades, few studies have focused on the development of hepatocyte serum-free medium. In this study, we developed a novel hepatocyte serum-free medium suitable for *in vitro* culture of C3A cells and applied an advanced method, gene-chip array, to explore the effect of sericin on the hepatocyte transcriptome. We found that sericin probably enhanced cell attachment through the CCR6-Akt-JNK-NF-κB pathway and promoted cell proliferation through CCR6-mediated activation of the ERK1/2-MAPK pathway. These findings inspired the following study on the mechanism by which sericin promotes cell attachment and proliferation.

INTRODUCTION

The bioartificial liver support system (BALSS) is a novel and ideal therapy for hepatic insufficiency, which can provide additional liver function for patients with acute liver injury and end-stage liver failure[1]. During the BALSS operation, hepatocytes in the bioreactor perform various functions such as albumin synthesis, ammonia elimination, and bilirubin metabolism, which can decrease the symptoms of liver failure[2].

The BALSS is mainly composed of a hepatocyte culture module and an extracorporeal circulation device[3]. At the present time, the cells used in BALSS are mainly primary porcine hepatocytes[4] and immortalized cells, such as HepG2 and C3A[5]. C3A is a human hepatocellular carcinoma cell line, with high albumin production and excellent ability of ammonia elimination. Therefore, C3A is selected as the hepatocyte in the extracorporeal liver assist device (ELAD), which has proven to be effective in liver support and biocompatible in patients in clinical trials[6].

Normally, *in vitro* culture of hepatocytes requires serum of animal-origin. However, the serum possesses several shortcomings, including immunogenicity, allergenicity and exposure to microorganisms[7]. During the operation of the BALSS, the hepatocyte culture medium is in contact with the patient's plasma in the bioreactor, resulting in the potential for a variety of adverse reactions such as anaphylaxis and bacteremia. Therefore, serum-free medium suitable for hepatocyte culture in the BALSS has been needed within recent decades. However, few studies have focused on this topic.

HepatoZYME-SFM, the most popular of all hepatocyte serum-free media, is a serum-free medium for the long-term maintenance of hepatocyte phenotypic expression including the active and inducible forms of cytochrome P450 and active phase Ⅱ enzymes[8]. However, it is mainly used for serum-free primary hepatocyte culture, and serum is required for the adherence of hepatocytes at the early stage of serum-free culture with HepatoZYME.

Generally, serum-free medium comprises nutrients, growth factors, adherence-promoting factors, hormones, and trace elements. Advanced DMEM/F-12 (Dulbecco's Modified Eagle Medium/Ham's F-12) is a widely used basal medium that allows the culture of mammalian cells with reduced (10-50 mL/L) fetal bovine serum (FBS) supplementation, so it is often selected as the basal medium of the serum-free medium. Growth factor is the key component of serum-free culture medium, as it promotes cell growth. Hepatocyte growth factor (HGF) is a key ligand that elicits G1/S progression of epithelial cells, including hepatocytes, by up-regulating cyclin-E1 *via* the proline-mTOR pathway[9]. Epidermal growth factor (EGF) is not only a promoter of the growth of epithelial cells but also an important regulator that promotes

CYP3A4 expression in hepatocytes[10]. Dexamethasone affects the growth of hepatocytes in a dose-dependent manner. HGF-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes are promoted by dexamethasone at the concentration of 10^{-10} mol/L, but are inhibited at the concentration of 10^{-8} mol/L[11]. Furthermore, dexamethasone effectively induces gluconeogenesis in malignant hepatocytes both *in vitro* and *in vivo* by up-regulating PEPCK and G6Pase expression[12].

Sericin, a silk-derived protein that constitutes 20%-30% of silk, is soluble in the water and envelops the fibroin fibers on the surface of silk. Sericin is widely used in the garment industry, cosmetics, pharmaceuticals, and biomedical engineering because of its moisture-regulating ability, ultraviolet (UV) resistance, and antibacterial, anticancer, and anticoagulant properties[13-15]. It is well known that sericin has the ability to promote the attachment and proliferation of several mammalian cells[16], and as a consequence, sericin has been widely used in cell cultures. In DMEM containing 100 mL/L FBS, cell viability and proliferation of normal animal cells, tumor cells, hybridoma cells and normal mouse fibroblast L929 cells were improved by adding sericin at a certain concentration[17].

Along with the development of culture technology, sericin has also been used for 3D cell culture. Mandal *et al*[18] reported that a novel biopolymeric matrix fabricated by chemically cross-linking polyvinyl alcohol (PVA) with silk sericin was superior to PVA with respect to swellability, mechanical strength and flexibility, and cell attachment and viability. In another study, a silk sericin/gelatin 3-D scaffold showed enhanced mechanical strength, and higher compressibility, swellability, and porosity than a 2-D film. Moreover, improved cell attachment and viability, and low immunogenicity have suggested that a sericin/gelatin 3-D scaffold may be an ideal biomedical material[19]. Sericin has also been used for hepatocyte culture and cryopreservation, the glucose consumption, urea secretion rate, and intracellular albumin content of HepG2 cells were increased in sericin-alginate-chitosan microcapsules[20]. Miyamoto *et al*[21] demonstrated that a serum-free solution containing sericin and maltose improved the attachment capability of cryopreserved primary hepatocytes.

Although sericin has been shown to promote cell attachment, viability, and proliferation, the mechanism has been clarified. It is probably that the promotion of viability and proliferation is based on enhanced cell attachment. Aramwit *et al*[22] showed that sericin had the capability to improve the production of type-I collagen in the mouse fibroblast cell line L929 and that sericin increased the production of collagen in a dose-dependent manner within the range of 0.2-1.0 mg/mL.

Despite the advantages of sericin, it has not been used in the development of serum-free medium. The purpose of this study was to develop a novel serum-free medium suitable for *in vitro* hepatocyte culture based on sericin, growth factors, and other additives. In addition, the effects of sericin on the gene expression of

hepatocytes were also investigated using a gene-chip array in order to explore the mechanism by which sericin promotes cell viability and proliferation.

MATERIALS AND METHODS

There were two parts to this study. Part 1 was a controlled trial comparing the novel serum-free medium to other media. Part 2 was a comparative study between serum-free medium with or without sericin.

Materials
Sericin, insulin-transferrin-sodium selenite liquid media supplement (ITS), and 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Advanced DMEM/F12, HepatoZYME, L-glutamine, FBS, and phosphate-buffered saline (PBS) were purchased from Thermo Fisher Scientific. Penicillin-streptomycin and 2.5 g/L trypsin-EDTA solution were purchased from Leagene. HGF, EGF, and dexamethasone were purchased from R&D Systems. Dimethyl sulfoxide (DMSO) was purchased from MP Biomedicals. A Live/Dead kit (calcein AM/PI) was purchased from Dojindo. SYBR Green qPCR SuperMix (a qPCR kit) was purchased from Invitrogen. TRIzol reagent and the RNeasy Plus Mini kit (reagents for extracting RNA from cells) were purchased from Ambion and Qiagen, respectively. An Olympus phase-contrast microscope and fluorescence microscope were used for cell observation. A Bio-Tek ELx800 absorbance reader was used for the MTT assay. An Aeroset automated biochemistry analyzer was used for the measurement of albumin and urea. A Bio-Photometer plus (Eppendorf) and an ABI PRISM®7500 Sequence Detection System (Thermo Fisher Scientific) were used for RT-qPCR. A BD FACSVerse was used for flow cytometry.

Preparation of medium
Sericin, HGF, and EGF were dissolved into PBS, and dexamethasone (Dex) was dissolved into DMSO at a certain concentration to create stock solutions, respectively. DMEM/F12 and HepatoZYME were employed as the basal medium for their corresponding group. The medium for each group in part 1 of the study was prepared by adding the supplements into the basal medium as shown in Table 1. In part 2, the medium used for group A was the same medium used for group A in part 1, while the medium used for group B was sericin knock-out (Table 2).

Cell lines and culture
The human hepatocellular carcinoma cell line C3A was obtained from ATCC (ATCC® CRL-10741). C3A cells were inoculated into 25 cm^2 flasks after thawing and cultured in DMEM containing FBS (100 mL/L) and penicillin-streptomycin (100 U/mL) under 50 mL/L CO_2 at 37 ℃. The medium was changed every two days. Once the cells reached confluence, they were harvested using 2.5 g/L trypsin-EDTA, followed by the addition of fresh culture medium to create a new single-cell suspension for further

Table 1 Medium formula of each group in part 1

Group A	Group B	Group C	Group D
DMEM/F12	HepatoZYME	DMEM/F12	DMEM/F12
ITS 10 mL/L	L-Glutamine 2 μmol/mL	FBS 100 mL/L	PEN-SM 100 U/mL
HGF 20 ng/mL	PEN-SM 100 U/mL	PEN-SM 100 U/mL	
EGF 10 ng/mL			
Dex 1 nmol/mL			
Sericin 2 mg/mL			
PEN-SM 100 U/mL			

ITS: Insulin-transferrin-sodium; HGF: Hepatocyte growth factor; EGF: Epidermal growth factor; Dex: Dexamethasone; PEN-SM: Penicillin-streptomycin; FBS: Fetal bovine serum.

Table 2 Medium formula of each group in part 2

Group A (sericin)	Group B (control)
DMEM/F12	DMEM/F12
ITS 10 mL/L	ITS 10 mL/L
HGF 20 ng/mL	HGF 20 ng/mL
EGF 10 ng/mL	EGF 10 ng/mL
Dex 1 nmol/mL	Dex 1 nmol/mL
Sericin 2 mg/mL	PEN-SM 100 U/mL
PEN-SM 100 U/mL	

ITS: Insulin-transferrin-sodium; HGF: Hepatocyte growth factor; EGF: Epidermal growth factor; Dex: Dexamethasone; PEN-SM: Penicillin-streptomycin; FBS: Fetal bovine serum.

incubation. The concentration of FBS was gradually reduced from 100 mL/L to 50 mL/L, then to 20 mL/L, and finally to 10 mL/L. After stable growth was achieved in DMEM containing 10 mL/L FBS, the cells were cultured in the corresponding medium for each group.

Live/dead fluorescence microscopy assay

Cell attachment after inoculation was evaluated by fluorescence microscopy using a Live/Dead kit. This method allows the simultaneous detection of both live and dead cells with calcein acetoxymethyl (calcein AM) and propidium iodide (PI) dyes. Calcein AM is a nonfluorescent and permeable reagent, which is converted by intracellular esterases to the intensely green fluorescent calcein. Propidium iodide enters dead cells through damaged membranes and produces a bright red fluorescence when bound to nucleic acids.

The cells were inoculated into 24-well plates at a density of 1×10^5 cells/well, and then cultured in the respective medium. Twenty-four hours later, the medium was discarded, and the cells were washed three times with PBS and incubated with calcein AM and PI under 50 mL/L CO_2 at 37 ℃ for 15 min. The morphology and quantity of the cells were observed using a fluorescence microscope at the wavelengths of 490 nm and 545 nm, respectively. The number of live cells in each photo was counted using the ImageJ software.

Cell growth curves

Cell growth was investigated every 24 h for seven days. Briefly, the cells were inoculated into 12-well plates at a density of 1×10^5 cells/well, and cultured in the respective medium under 50 mL/L CO_2 at 37 ℃. Every 24 h, the medium was changed, and the cells in each group were counted using a phase-contrast microscope in triplicate.

Cell viability and proliferation assessment

The viability and the proliferation capacity of the cells were quantitatively assessed by MTT assay. This assay is based on the reduction of MTT (a tetrazolium salt solution) to purple formazan by metabolically active cells. For this analysis, 5×10^3 cells were inoculated into each well of 96-well plates, and subsequently cultured in the respective medium under 50 mL/L CO_2 at 37 ℃ for seven days. Every 24 h, the cells were incubated with 1 mg/mL MTT for 4 h. After solubilization in DMSO for 10 min, the concentration of the formazan produced by the metabolically active cells was quantified at 490 nm with a Bio-Tek ELx800 absorbance reader in quintuplicate.

Cell function assessment

Cell function was assessed by the amount of albumin and urea produced by metabolically active cells. The cells were inoculated into 24-well plates at a density of 1×10^5 cells/well and cultured in the respective medium under 50 mL/L CO_2 at 37 ℃ for seven days. The supernatant was collected every 24 h for quantitative testing of albumin and urea using an Aeroset automated biochemistry analyzer in triplicate.

Biocompatibility assessment for mediums

The biocompatibility of the media was evaluated by aspartate transaminase (AST) and lactate dehydrogenase (LDH), which were released into the culture medium by the cells through damaged membranes after suffering an acute injury. Their concentrations in the culture media are correlated with membrane damage and the biocompatibility of the medium.

Every 24 h post-seeding, the supernatant was harvested for quantitative testing of AST and LDH using an Aeroset automated biochemistry analyzer in triplicate for seven days.

Cell cycle analysis

Cells were inoculated into 6-well plates at a density of 5×10^5 cells/well, and cultured in the respective medium

under 50 mL/L CO_2 at 37 ℃. Seventy-two hours later, the cells were detached by trypsinization, centrifuged, and the pellet of cells was immediately fixed with ice-cold 700 mL/L ethanol. Afterwards, cells were washed three times with cold PBS to remove the ethanol, and finally stained with PI using a standard method[23]. Cells were analyzed by flow cytometry using BD FACSVerse.

Gene chip array

The differences in the transcriptomes of the cells cultured in the presence of sericin were quantitatively assessed using the GeneChip® PrimeView™ Human Gene Expression Array. The cells were inoculated into 6-well plates at a density of 1.5×10^6 cells/well, and subsequently cultured in the respective medium under 50 mL/L CO_2 at 37 ℃. Seventy-two hours post seeding, the cells were harvested and total RNA was extracted using a Qiagen RNeasy Plus Mini kit. After the quality was verified using a Thermo Nanodrop 2000 and Agilent 2100 Bioanalyzer, the RNA was analyzed using GeneChip® PrimeView™ to reveal the differentially expressed genes related to proliferation. The expression differences of up-regulated genes were presented as fold-change (group A/group B ratio), and differences between samples were considered statistically significant at a value of fold-change > 1.5.

Real time quantification of gene expression

RT-qPCR was used to distinguish the differences of genes related to hepatocyte function in each group of part 1, and to verify the differentially expressed genes in the gene chip array of part 2. The cells were inoculated into 6-well plates at a density of 1.5×10^6 cells/well, and subsequently cultured in the respective medium under 50 mL/L CO_2 at 37 ℃. At each set time point, the cells were harvested and total RNA was extracted using a Qiagen RNeasy Plus Mini kit. After RNA quality verification, RT-qPCR was performed using an ABI PRISM®7500 Sequence Detection System and SYBR Green qPCR SuperMix. The genes related to hepatocyte function were assessed two, four, and six days after seeding, and included uridinediphosphate-glucuronosyl transferase (UGT: The enzyme catalyzing the conversion of unconjugated bilirubin into conjugated bilirubin), glutathione S-transferase (GST: The enzyme catalyzing the conjugation of the reduced form of glutathione to xenobiotic substrates for the purpose of detoxification), glutamate-ammonia ligase (GLUL: The key enzyme in glutamine synthesis), glucose-6-phosphatase (G6P: The key enzyme in gluconeogenesis and glycogenolysis), albumin, carbamoyl phosphate synthetase Ⅰ (CPS1: The key enzyme in the production of urea), and cytochrome P-450 (CYP3A4, CYP2D6: the oxidizing enzymes in drug metabolism). The genes screened by the gene chip array were assessed one, two, three, and four days after seeding. The relative expression of each gene was presented as the value of $2^{-\triangle\triangle Ct}$.

Statistical analysis

The statistical analyses of the data in parts 1 and 2

were performed using one-way ANOVA and a *t*-test, respectively, with SPSS version 20.0 software. The results were expressed as the mean ± standard deviation (SD) using GraphPad Prism Software. Differences between samples were considered statistically significant at a value of $P < 0.05$.

RESULTS

Part 1

In part 1, group A is our novel serum-free medium, group B is HepatoZYME, group C is the complete medium (DMEM/F12 with 100 mL/L FBS), and group D is DMEM/F12 (Table 1).

Live/dead fluorescence microscopy assay: Cell behavior in terms of attachment and viability was qualitatively investigated after 24 h of culture under standard conditions by fluorescence microscopy, based on the simultaneous staining of live (green-labeled) and dead (red-labeled) cells (Figure 1).

The density of attached living cells in group A was obviously higher than those in group B and group D, and almost approached that of group C. C3A cells in group A possessed a greater attachment capability than those in group B and group D ($P < 0.01$).

Cell growth curves: Cell growth was directly measured by counting the cell number in each group with standard hemocytometry every 24 h. During the seven days post-seeding, the cell number in each group increased gradually at different rates (Figure 2A). Within three days post-seeding, the difference in the cell numbers among groups A, B and C were not significant. Beginning on the fourth day post-seeding, the cell number in group A was significantly higher than those in group B and group D at each time point ($P < 0.001$). Although the daily cell number in group A was lower than that in group C starting on the fifth day post-seeding ($P < 0.001$), they were close in number during the first four days of culture.

Cell viability and proliferation assessment: To examine cell viability and the proliferation rate, the MTT assay was employed in which the number of metabolically active cells is linearly associated with the absorbance at 490 nm within a certain range. During the whole culture period, the curve of each group rose gradually at different rates (Figure 2B). Within three days post-seeding, the cell viability and proliferation of group A were similar to group C. Afterwards, the number of metabolically active cells in group A was less than that in group C ($P < 0.001$), and reached the maximum at day 5. The viabilities of groups A, B and D declined after day 5. Nevertheless, the daily number of metabolically active cells in group A was significantly greater than those in group B and group D during the whole culture period ($P < 0.001$).

Cell function assessment: Cell function was assessed by the quantity of albumin and urea produced by C3A
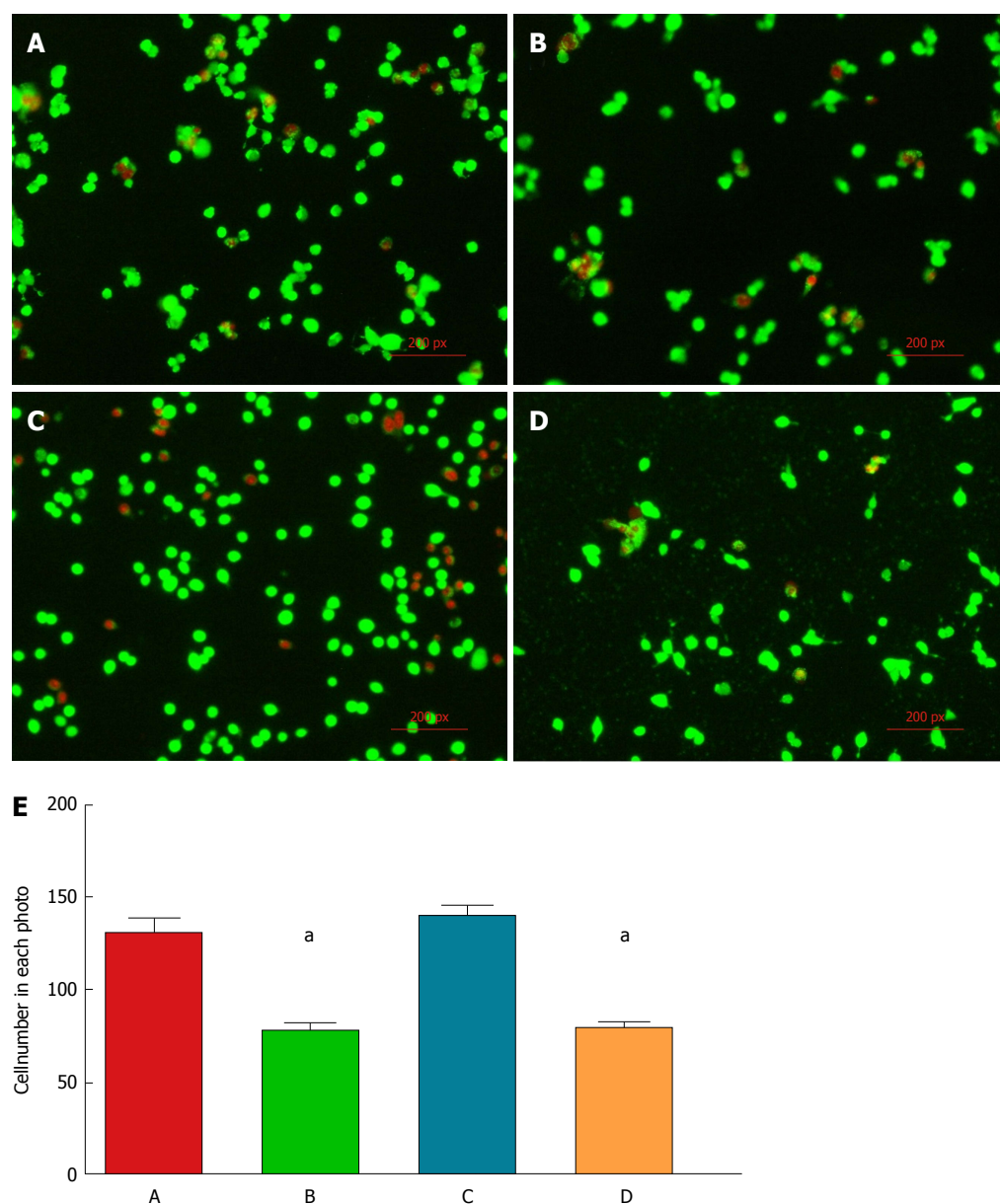
Figure 1 Fluorescence microscopy assessment of live (green-labeled) and dead (red-labeled) C3A cells at 24 h post-seeding in each group. A: Serum-free medium; B: HepatoZYME; C: DMEM/F12 with 100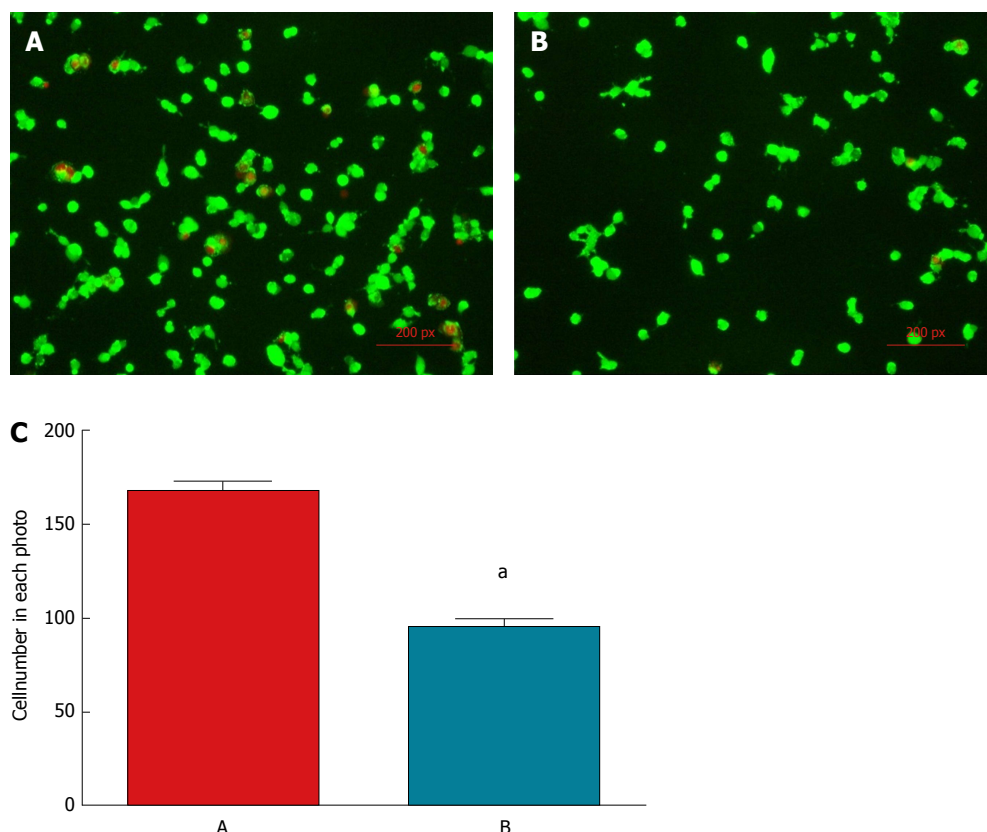 mL/L FBS; D: DMEM/F12; and E: comparison of cell number from photos in each group: (group A) serum-free medium, (group B) HepatoZYME, (group C) DMEM/F12 with 100 mL/L FBS, and (group D) DMEM/F12. Data are expressed as mean ± SD (n = 3). [a]P < 0.05. FBS: Fetal bovine serum.

cells. With respect to albumin (Figure 2C), the curves of group A and group C showed a slightly declining trend throughout the whole culture period. On days 1, 2, 6, and 7, the albumin in the supernatant of group A was lower than that in group C (P < 0.001), but there was no significant difference on days 3, 4, and 5. Throughout the culture process, the quantity of albumin secreted by C3A cells in group A was greater than that in group B and group D at each time point (P < 0.001). With respect to urea (Figure 2D), the content of urea in the supernatant of each group decreased gradually during the culture period. At each time point, the urea synthesized by the C3A cells in group A was lower than that in group C (P < 0.001), but it was significantly greater than that in group B and group D (P < 0.001).

Biocompatibility assessment for media: As they reflect hepatocyte injury, AST and LDH were selected to assess the biocompatibility of each medium with C3A cells. With respect to AST leakage (Figure 2E), there were no differences among the groups during the first 24 h. Beginning on day 2, AST leakage in groups A, C, and D was similar until day 6, and significantly lower than that in group B at each time point (P < 0.001). AST leakage in group A was lower than that in all the other groups on day 7 (P < 0.001). With respect to LDH leakage (Figure 2F), on days 1, 2, 5, and 6, LDH leakage in group A was greater than that in group C, but they shared a similar level at the other time points. During the whole culture period, LDH leakage in group B was significantly greater than that in group A and group C (P

Figure 2 Comparison on (A) cell growth, (B) cell viability, (C) ALB secretion, (D) urea production, (E) AST leakage and (F) LDH leakage in each group. (group a) Serum-free medium, (group b) HepatoZYME, (group c) DMEM/F12 with 100 mL/L FBS, (group d) DMEM/F12. Data are expressed as mean ± SD (n = 5 in B and 3 in the others). e: Significant difference between group A and group B at the same incubation time. f: Significant difference between group A and group C at the same incubation time. α: Significant difference between group A and group D at the same incubation time. $P < 0.05$. AST: Aspartate transaminase; LDH: Lactate dehydrogenase; FBS: Fetal bovine serum.

< 0.001). In the early stage of culture, the LDH content in the supernatant of group D was greater than that in group A and group C, and unexpectedly, LDH levels in group D was the lowest from day 4 to day 6 ($P < 0.001$).

Expression of genes related to hepatocyte functions: The differences in gene expression related to hepatocyte functions were estimated by using RT-qPCR, a highly sensitive and effective method to detect the expression of specific genes. Uridinediphosphate-glucuronosyl transferase (*UGT*) (Figure 3A): On day 2 and day 6, the relative expression in group A was lower than that in group C, but higher than that in group B and group D ($P < 0.001$). On day 4, the relative expression in group A was slightly lower than that in group B and

group C, but still higher than that of group D ($P < 0.001$). Glutathione S-transferase (*GST*) (Figure 3B): At each time point, the relative expression of group A was lower than that of group B and group C, but greater than that of group D ($P < 0.001$). Glutamate-ammonia ligase (*GLUL*) (Figure 3C): The relative expression in group C was higher than that in the other groups at all time points. The relative expression in group A was lower than that in group B at day 2 and 4, but significantly higher at day 6 ($P < 0.002$). Glucose-6-Phosphatase (*G6P*) (Figure 3D): At each time point, the expression in group A was lower than that in group B and group C, but higher than that in group D ($P < 0.001$). Albumin (Figure 3E): The relative expression in group A was lower than that in group C, but significantly higher than that in the other

Figure 3 Relative expression of genes related to hepatocyte function. A: *UGT*; B: *GST*; C: *GLUL*; D: *G6P*; E: *albumin*; F: *CPS1*; G: *CYP3A4*; and H: *CYP2D6* in each group. (group a) Serum-free medium, (group b) HepatoZYME, (group c) DMEM/F12 with 100 mL/L FBS, (group d) DMEM/F12. Data are expressed as mean ± SD (n = 3). [e]P < 0.05, significant difference from group a at the same incubation time. *UGT*: Uridinediphosphate-glucuronosyl transferase; *GLUL*: Glutamate-ammonia ligase; *CPS1*: Carbamoyl phosphate synthetase I; *G6P*: Glucose-6-phosphatase; *CYP3A4*: Cytochrome P3A4; *CYP2D*: Cytochrome P2D6; FBS: Fetal bovine serum.

groups (P < 0.001). Carbamoyl phosphate synthetase 1 (*CPS1*) (Figure 3F): The expression in group A was greater than that in group B and group D, although lower than that in group C (P < 0.001). Cytochrome P 3A4 (*CYP3A4*) (Figure 3G): At each time point, the expression in group A was greater than that in group B and group D (P

Figure 4 Fluorescence microscopy assessment of live (green-labeled) and dead (red-labeled) C3A cells 24 h post-seeding in each group. (A) serum-free medium containing 2 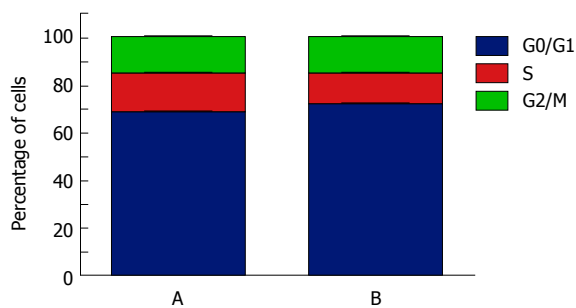mg/mL sericin, (B) serum-free medium without sericin, (C) Comparison of cell number from photos in each group: (group A) Serum-free medium containing 2 mg/mL sericin, (group B) serum-free medium without sericin. Data are expressed as mean $\pm$ SD (n = 3). [a]P < 0.05, significant difference between group A and group B at the same incubation time.

< 0.001). The expression in group A was lower than that in group C on days 2 and 4, but higher than that in the other groups on day 6 (P < 0.001). Cytochrome P 2D6 (*CYP2D6*) (Figure 3H): On day 2 and 6, the expression in group A was significantly higher than that in the other groups (P < 0.001); however, there were no significant differences on day 4.

Part 2

Live/dead fluorescence microscopy assay: The attachment capability of C3A cells was evaluated by the amount of live cells adhering to the plate (green-labeled) 24 h post-seeding. In the presence of sericin, a larger number of cells adhered to the surface (P < 0.01), and afterwards proceeded with proliferation as well as normal physiological functions (Figure 4).

Cell growth curves: Daily cell counting revealed that the cell growth curves of both groups rose gradually in the early stage of culture, then reached a plateau at day 5 (Figure 5A). At every time point, the cell growth in group A was superior to that in group B (P < 0.001).

Cell viability and proliferation assessment: The absorbance curves in both groups rose gradually wi-thin four days post-seeding (Figure 5B). On day 5, the viability of group A reached a peak value and then declined slightly, while that of group B reached a plateau. Over the whole culture period, there were more meta-bolically active cells in group A than in group B (P < 0.001).

Cell cycle analysis: The cell cycle was divided as G0/G1, S, and G2/M phase, and S phase was the DNA synthesis period. Therefore, the percentage of cells in S phase of the whole cell population was used to evaluate cell proliferation. The cell cycle analysis indicated that the proportion of cells in S phase in group A was 16.21% $\pm$ 0.98%, while that in group B was 12.61% $\pm$ 0.90% (P = 0.009) (Figure 6). These results indicated that the cells in group A possessed a stronger proliferative capability than those in group B.

Gene chip array: Since the previous experiments verified that the attachment and proliferative capabilities of C3A cells were significantly enhanced by 2 mg/mL sericin, we decided to proceed with exploring the impact of sericin on the C3A transcriptome. The differential gene expression profile between the two groups was analyzed by gene chip array, and the expression differences in up-regulated genes were presented as fold-change (group A/group B ratio). A cut-off of > 1.5-fold increase was applied, and the results revealed that a total of 250 genes were significantly up-regulated by sericin.

Figure 5 Comparison of (A) cell growth curve and (B) cell viability curve of each group. (group a) Serum-free medium containing 2 mg/mL sericin, (group b) serum-free medium without sericin. Data are expressed as mean ± SD (*n* = 3 and 5, respectively). [c]*P* < 0.05, significant difference between group a and group b at the same incubation time.

Figure 6 Cell cycle distribution of each group. (group A) Serum-free medium containing 2 mg/mL sericin and (group B) serum-free medium without sericin. Data are expressed as mean ± SD (*n* = 3). The portion of cells in S phase in group A (16.21% ± 0.98%) was significantly higher than that in group B (12.61% ± 0.90%) (*P* = 0.009).

To characterize the signaling pathways modulated by sericin in C3A cells, a pathway enrichment analysis was conducted using Ingenuity Pathway Analysis (IPA) according to the KEGG and BIOCARTA databases. The top significantly enriched pathways modulated by sericin were determined (Figure 7), among which KEGG-pathways in cancer and the MAPK signaling pathway were associated with cell proliferation. The genes related to cell proliferation were *COL4A6*, *MAX*, *EGFR*, *FOS*, *CDC42*, *FGF12*, and *SLC2A1* within the pathways in cancer, and *EGFR*, *FOS*, and *MAPK13* within the MAPK signaling pathway. The classic MAPK signaling pathway is included in the pathways in cancer, so the genes included in both pathways, *EGFR* and *FOS* (Table 3), were considered to be the genes affecting the proliferation of C3A cells with the most potential.

Subsequently, the other significantly up-regulated genes in group A in comparison with group B were screened by literature review to determine the genes related to proliferation. It has been demonstrated that *CCR6* and the MAPK pathway are associated with the migration and proliferation of breast epithelial cells. Thus, RT-qPCR was used to verify the gene expressions of *CCR6*, *EGFR*, and *c-FOS*, as well as the molecules in the MAPK pathway, including *Src*, *PI3K*, *AKT1*, *JNK1*, *NFkB1*, *MMP-9*, *GRB2*,

SHC2, *K-RAS*, *RAF1*, *MEK2*, *ERK1/2*, *c-myc* and *cyclinE1*.

mRNA expression of CCR6 and the MAPK pathway: The early stage is the key period for attachment and proliferation, so RT-qPCR was performed on the samples at days 1, 2, 3, and 4 post-seeding. The expression difference at each time point was presented as fold-change [fold-change = $2^{-(\Delta Ct\ groupA\ -\ \Delta Ct\ groupB)}$] in Table 4. *CCR6*: The expression of *CCR6* in group A was greater than that in group B from inoculation until day 3 (*P* < 0.02), and *CCR6* showed a 2.36-fold increase in group A compared to group B on day 3. *EGFR*: The expression in group A was greater at all time points (*P* < 0.01), and the increases of *EGFR* in group A were 7.71- and 2.34-fold compared to group B on days 2 and 4, respectively. *FOS*: On day 1 and day 3, the expression in group A was higher than that in group B (*P* < 0.001). *Src*: Beginning on day 2, the mRNA expression in group A was significantly higher than that in group B (*P* < 0.001), and the increases of *Src* in group A were 2.44- and 3.67-fold compared to group B on days 2 and 4, respectively. *PI3K*: The significant increases in expression in group A ranged from 3.00- to 3.10-fold compared to group B on days 1, 2 and 4 (*P* < 0.001). *AKT1*: At each time point, the expression in group A was greater than that in group B (*P* < 0.01), and the increase of *AKT1* in group A was 2.04-fold compared to group B on day 3. *JNK1*: In the early stage of culture, the expression of the two groups was similar, but the expression in group A significantly increased 2.69- and 2.09-fold compared to group B on days 3 and 4 (*P* < 0.001). *NFkB1*: The expression in group A was greater than that in group B on days 2 and 3 (*P* < 0.001). *MMP-9*: The expression was greater in group A at all time points except on day 2 (*P* < 0.006). *GRB2*: On days 2 and day 3 the expression was significantly greater in the presence of sericin (*P* < 0.001). *SHC2*: Beginning on day 3, the expression was greater in group A (*P* < 0.001). *K-RAS*: The expression in group A was higher than that in group B at each time point except on day 1 (*P* < 0.01). *RAF1*: Beginning on day 2, the expression in group A was greater than that
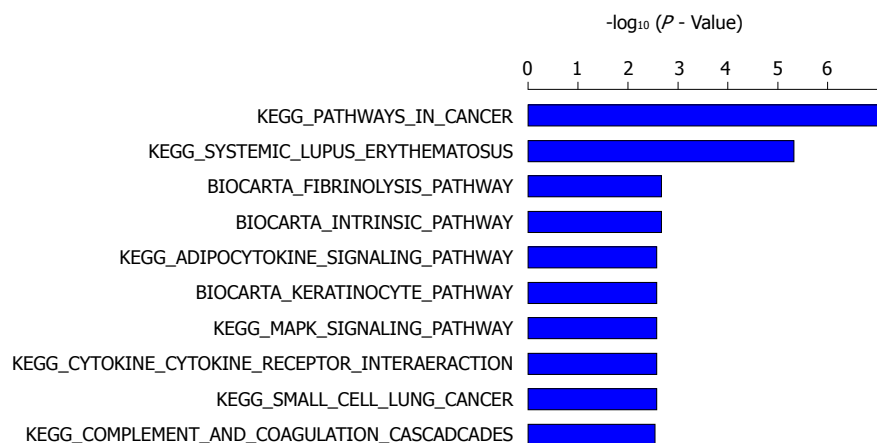
Table 3 Up-regulated genes associated with cell proliferation

Up-regulated genes	Pathways involved	Fold change
CCR6	Cytokine-cytokine receptor interaction	1.99
EGFR	Pathways in cancer, MAPK signaling pathway	1.55
FOS	Pathways in cancer, MAPK signaling pathway	1.59

Table 4 Comparison of *CCR6* expression and genes in the MAPK pathway

Genes	FC in gene chip	FC1	FC2	FC3	FC4
CCR6	1.99	1.36 ± 0.09^1	1.40 ± 0.01	2.36 ± 0.04^1	0.75 ± 0.01
EGFR	1.55	1.76 ± 0.04^1	7.71 ± 0.49^1	1.46 ± 0.13^1	2.34 ± 0.24^1
FOS	1.59	1.22 ± 0.09^1	0.96 ± 0.07	1.77 ± 0.05^1	0.96 ± 0.01
Src	1.15	0.84 ± 0.16^1	2.44 ± 0.08^1	1.93 ± 0.70^1	3.67 ± 0.40^1
PI3K	1.24	3.00 ± 0.27^1	3.01 ± 0.39^1	1.43 ± 0.15	3.10 ± 0.14^1
AKT1	1.13	1.22 ± 0.04^1	1.21 ± 0.05^1	2.04 ± 0.10^1	1.59 ± 0.06^1
JNK1	1.09	0.98 ± 0.07	1.07 ± 0.11	2.69 ± 0.09^1	2.09 ± 0.04^1
NFκB1	1.03	0.97 ± 0.07	1.51 ± 0.02^1	1.77 ± 0.04^1	0.84 ± 0.02
MMP-9	1.22	1.38 ± 0.15^1	1.10 ± 0.06	1.51 ± 0.04^1	1.56 ± 0.04^1
GRB2	1.05	1.04 ± 0.10	1.48 ± 0.03^1	1.34 ± 0.01^1	1.06 ± 0.05
SHC2	1.30	0.98 ± 0.03	1.13 ± 0.06	1.39 ± 0.21^1	1.47 ± 0.17^1
K-RAS	1.09	1.16 ± 0.05	1.21 ± 0.08^1	1.42 ± 0.04^1	1.53 ± 0.01^1
RAF1	1.01	1.09 ± 0.10	1.24 ± 0.02^1	1.48 ± 0.02^1	1.22 ± 0.03^1
MEK2	1.02	5.86 ± 1.22^1	4.61 ± 1.11^1	2.38 ± 1.05^1	8.45 ± 1.00^1
ERK1	1.07	1.80 ± 0.62^1	1.29 ± 0.03^1	1.02 ± 0.26	1.19 ± 0.02
ERK2	1.18	2.58 ± 0.21^1	2.52 ± 0.62^1	5.15 ± 0.26^1	1.63 ± 0.06^1
c-myc	1.17	2.32 ± 0.21^1	1.67 ± 0.27^1	1.41 ± 0.30^1	0.06 ± 0.01^1
cyclinE1	1.04	1.04 ± 0.13	1.52 ± 0.03^1	2.21 ± 0.02^1	1.48 ± 0.01^1

[1]Significant difference between group A and group B at the same incubation time ($P < 0.05$). FC1, FC2, FC3, FC4 was the fold change (group A/group B) of gene expression measured by RT-qPCR on days 1, 2, 3, 4, respectively. Data are expressed as mean $\pm$ SD (n = 3).

Figure 7 Pathway enrichment in the gene chip array.

in group B ($P < 0.001$). *MEK2*: The significant increase in expression in group A ranged from 2.38- to 8.45-fold compared to group B ($P < 0.001$). *ERK1*: The expression of *ERK1* in group A was greater than that in group B on the first two days ($P < 0.001$). *ERK2*: The expression was greater in group A at every time point ($P < 0.001$), and the fold change ranged from 1.63 to 5.15. *c-myc*: In the first three days, the mRNA expression of *c-myc* was higher in the presence of sericin ($P < 0.001$), and the fold change on day 1 was 2.32. *cyclinE1*: The expression in group A was greater except on day 1 ($P < 0.001$), and the fold change on day 3 was 2.21.

DISCUSSION

Serum is a necessary supplement for the *in vitro* culture of mammalian cells, because of its capability to simulate a suitable microenvironment similar to that of *in vivo* culture. However, serum is always removed from the culture for certain purposes, such as avoiding the impacts of serum on the experiment results and immunological rejection in heterogeneous animal *in vivo* experiments. To maintain normal attachment, proliferation, synthesis, and other cellular physiological processes, serum-free medium needs to contain a number of essential factors

including basic nutrients, attachment promoters, growth factors, trace elements, and hormones[24,25].

In previous experiments, we selected several biomaterials including sericin, HGF, EGF, and dexamethasone as supplements in serum-free medium, followed by exploring their optimal concentrations in the serum-free medium. Finally, the most suitable serum-free medium was successfully prepared for subsequent controlled trials, and included HepatoZYME, DMEM/F12 and DMEM/F12 supplemented with 100 mL/L FBS (complete medium).

Sericin, the "glue" on the surface of the silk fibers, has been proven to be one of the perfect substitutes for serum[26]. Sericin has the ability to promote the attachment and proliferation of mammalian cells[27]. Our experiment exploring the best concentration of sericin in serum-free culture medium indicated that C3A cells proliferated at the highest rate in the medium containing 2 mg/mL sericin. In contrast, the C3A cells in the medium containing 5 mg/mL sericin exhibited growth arrest. This result corresponded to that reported by Terada *et al*[16], which demonstrated that an overload of sericin was harmful to the cells.

In this study, cell attachment, cell viability and proliferation, hepatocyte function, biocompatibility of the medium with the cells, and the expression of genes related to hepatocyte functions were examined in the different media. For hepatocytes, attachment to a carrier is necessary for biological processes. During the first 24 h after inoculation, a larger number of C3A cells adhered to the surface of the plate in our serum-free medium compared with HepatoZYME and DMEM/F12. In addition, the adherence approached that observed for a complete medium. The excellent adherence of C3A cells in our serum-free medium can be mostly attributed to the attachment-promoting capability of sericin, which has been shown in a number of other studies. Akturk *et al*[28] demonstrated that a sericin/collagen membrane had the ability to enhance the initial attachment of keratinocytes 24 h post-seeding, and adhesion proteins such as collagen, laminin, and fibronectin were also present in their sericin/collagen membrane, which even contained the arginine-glycine-aspartic (RGD) acid sequence recognized by the cell surface receptors integrins.

During the culture period, the daily cell number in our serum-free medium was similar to that in the complete medium in the early period, and greater than that in HepatoZYME and DMEM/F12 during the whole culture period. Furthermore, this result was consistent with the cell viability and proliferation assessment. As previously mentioned, sericin promotes cell proliferation, making our serum-free medium superior to other media with respect to cell growth. Although HGF and EGF are also mitogens for hepatocytes, the results of the experiments in part 2 indicated that sericin was the dominant growth promoter in our serum-free medium. However, in the later stage of culture, the viability of hepatocytes in the serum-free medium declined slightly, indicating that the promotion of proliferation by the serum-free medium on

hepatocytes was a short-term (within the first four-five days of culture) effect.

Biocompatibility with the cells, tissues, and organs is an important evaluation standard for biomaterials. Sericin has been proven to be a biocompatible material for a variety of cells, as it does not cause cell cycle arrest and it releases few inflammatory mediators[19,29]. AST and LDH concentrations in the supernatant are commonly employed as indicators of acute hepatocyte damage because of their leakage from hepatocytes after injury. In this study, the AST concentration in our serum-free medium and the complete medium were similar during almost the whole culture period, and significantly lower than that in HepatoZYME at each time point. Similarly, LDH leakage in the serum-free medium was less than that in HepatoZYME. These results indicated that there was excellent biocompatibility of our serum-free medium with hepatocytes. However, low AST leakage should be attributed to not only the excellent biocompatibility of sericin but also the protection of HGF. Glanemann *et al*[30] demonstrated that pretreatment with HGF significantly reduced AST leakage in rat hepatocytes and reactive oxygen intermediate formation by increasing glutathione synthesis during inflammation.

As the core of the BALSS, the hepatocyte functions attract the most attention. In this study, biochemical assays and RT-qPCR were employed to evaluate hepatocyte functions. The albumin synthesis in the serum-free medium was similar to that in the complete medium in the middle of the culture period, and greater than that in HepatoZYME and DMEM/F12 at every time point. This result was consistent with the mRNA expression of albumin assessed by RT-qPCR. However, during long-term culture, the albumin synthesized by C3A cells was on a downward trend. In other words, the hepatocytes used in BALSS should be replaced every four-five days in order to ensure persistent albumin synthesis. The eximious ALB synthesis was associated with HGF in our serum-free medium, as shown by Hou *et al*[31]. Hou *et al*[31] manufactured a HGF/heparin-immobilized collagen system as a synthetic extracellular matrix for hepatocyte culture, in which albumin synthesis was greater than that in heparin-immobilized collagen, revealing that HGF promotes albumin synthesis in hepatocytes.

Though lower than that in complete medium, urea production in our serum-free medium was significantly greater than that in HepatoZYME and DMEM/F12, as indicated by both the quantitative detection of urea and the mRNA expression of *CPS1*.

UGT is the primary phase II enzyme catalyzing the conjugation of glucuronic acid to the xenobiotics, with polar groups facilitating their clearance[32]. The *UGT* expression in our serum-free medium was greater than that in HepatoZYME and DMEM/F12, and approached that in complete medium. CYP450 are phase I enzymes responsible for the metabolism of at least 90% of drugs[33], among which CYP3A4 and CYP2D6 are the most important. Our study showed excellent CYP450 expression in the serum-free medium, close to

and even sometimes superior to that in the complete medium. Dexamethasone is considered an inducer of CYP3A4[34]. In sheep small intestine, dexamethasone caused a significant enhancement of CYP3A apoprotein level in the duodenal mucosa[35]. Thus, we suggest that dexamethasone played an important role in the excellent CYP450 expression in the serum-free medium. Although it was shown that dexamethasone could effectively up-regulate G6Pase expression[12], the result in our study was the opposite, probably because of the different concentration of dexamethasone used in the studies.

Compared with HepatoZYME and DMEM/F12, our serum-free medium exhibited advantages in C3A cell attachment, proliferation, functions, and biocompatibility, making it a perfect medium for serum-free hepatocyte culture.

Since the C3A cells exhibited excellent proliferation in our serum-free medium, the key factor, sericin, became the focus of the study. In part 2, we designed a controlled trial with two groups: the serum-free medium with or without sericin. Twenty-four hours after inoculation, more C3A cells were attached to the surface of the plate in the presence of 2 mg/mL sericin, followed by better proliferation and viability during the whole culture period, which corroborated the conclusions of other studies[19,20,36]. Based on its positive effects on cell proliferation, sericin has been added to antimicrobial creams and wound dressings to ameliorate wound healing[28,37].

Considering that sericin promotes cell proliferation, the experiments in part 2 were designed to explore the underlying mechanisms. As a key cyclin for liver regeneration[38], cyclinE1 is an important regulator of G1/S progression in hepatocytes. Up-regulated cyclinE1 could prompt cells to proceed from G1 phase to S phase, when DNA is synthesized in a large amount for the subsequent mitosis[9]. Our study clarified that the percentage of S phase cells was elevated in the presence of sericin, while that of cells in G0/G1 phase was reduced accordingly. In addition, the expression of *cyclinE1* was significantly higher in the presence of sericin, indicating that the promotion of sericin for cell proliferation might result from active G1/S progression mediated by cyclinE1.

To explore the mechanism by which sericin promotes cell proliferation, we performed gene chip array analysis to distinguish the differences in the transcriptome between the two groups. Among the most significantly up-regulated genes, *CCR6*, *EGFR*, and *FOS* were implicated in cell proliferation.

Chemokine receptor 6 (CCR6) is the unique receptor of chemokine (C-C motif) ligand 20 (CCL20), which has been proven to promote the proliferation of malignant cells[39]. The up-regulation of *CCR6* promotes spontaneous intestinal tumorigenesis[40]. Brand *et al*[41] demonstrated that *CCR6* mediated the activation of *Akt*, *ERK-1/2*, and *SAPK/JNK* MAP kinases, resulting in increased intestinal epithelial cell migration and proliferation. In breast epithelial cells, CCL20/CCR6 binding promotes cell migration and proliferation by activating the

MAPK pathway[42]. Fujii *et al*[43] also reported that CCL20 enhanced the growth of HuH7 cells *via* phosphorylation of p44/42 MAPK *in vitro*. As signaling molecules in the MAPK pathway, up-regulated *EGFR* and *c-FOS* have been demonstrated to be promotors of proliferation. EGFR is the receptor for EGF, the phosphorylation and activation of which induces proliferation of several cells[44,45]. Mitogenic effects of many substances are mediated by *c-FOS*[46], and its overexpression could increase the proliferation of human hepatocytes by stabilizing nuclear Cyclin D1[47], while its blockage inhibits the proliferation and invasion of cancer cells[48].

Based on the gene chip array results and conclusion of the other studies, we assessed *CCR6* and molecules in the MAPK pathway by RT-qPCR to identify differential expression between the two groups. As a transmembrane receptor, the expression of *CCR6* was up-regulated in the early post-seeding period, resulting in increased expression of molecules in the MAPK pathway at different time points. At the transcription level, it was speculated that sericin promoted attachment and proliferation by two pathways: CCR6 on the C3A cell membrane was activated in the presence of sericin; subsequently the signal was transduced through the activation of *Src*, *PI3K*, *AKT1*, *JNK1* and *NFkB1*, and finally *MMP-9* was up-regulated. *MMP-9* is associated with cell migration because of its capability to remodel the extracellular matrix[49]. *MMP-9* up-regulation through a *Src*-dependent pathway is consistent with previous studies showing that oleic acid and denatured type-IV collagen induced *MMP-9* secretion and invasion in breast cancer cells[50,51]. The enhanced attachment of C3A cells by sericin was supposed to result from the increased migration. On the other hand, the CCL20/CCR6 binding transactivated the EGFR on the membrane, leading to a chain up-regulation of *GRB2*, *SHC2*, *K-RAS*, *RAF1*, *MEK2* and *ERK1/2*. Furthermore, ERK1/2 translocates into the nucleus and enhances transcription of early-response genes including *c-myc* and *c-FOS*[52]. Subsequently, the up-regulation of *c-myc* and *c-FOS* increased the expression of *cyclinE1*, promoting the G1/S progression and proliferation of C3A cells. However, this inference has not been proven by protein expression, so more effort should be made to clarify the mitogenic mechanism of sericin.

There are some limitations in this study. Although it is proven that our serum-free medium is suitable for *in vitro* culture of C3A cells, and sericin promotes the attachment and proliferation of C3A cells, it is still not clear whether the results obtained are restricted to C3A cells. In the further study, we are going to verify whether this serum-free medium is suitable for other hepatocytes, and determine the effect of sericin on other hepatocytes, such as HepG2, HuH7 and primary porcine hepatocytes. Hyperammonemia and hyperbilirubinemia are the clinical features of patients with liver failure. However, the urea production under an overload of NH_4^+ and the ability to convert non-conjugated bilirubin into conjugated bilirubin of hepatocytes were not assessed in this study. These functions of hepatocytes will be assessed in the further

study.

In summary, a novel serum-free medium for hepatocytes was developed in this study, and it exhibits excellent biocompatibility, and an enhanced capability of promoting cell attachment and proliferation, and provides a suitable microenvironment for hepatocyte functioning. It raises the possibility of large-scale serum-free culture of hepatocytes in the BALSS. In addition, the mechanism by which sericin promotes cell proliferation was explored, and it is speculated that sericin enhances cell attachment through the CCR6-Akt-JNK-NF-κB pathway, and promotes cell proliferation through CCR6-mediated activation of the ERK1/2-MAPK pathway.

ARTICLE HIGHLIGHTS

Research background
A serum-free medium suitable for hepatocyte culture in the bioartificial liver support system (BALSS) has been needed within recent decades, but few studies have focused on the development of hepatocyte serum-free medium. Sericin was proven to promote cell attachment and proliferation, but the mechanism is not clarified.

Research motivation
Poor adherence and proliferation were often observed in the serum-free culture. Sericin has the ability to promote the attachment and proliferation of several mammalian cells, so it was selected as a key supplement in our serum-free medium. The mechanism how sericin promotes the attachment and proliferation of hepatocytes was not clarified. So, the effect of sericin on the hepatocyte transcriptome was explored in this study.

Research objectives
To develop a novel serum-free hepatocyte medium and to clarify the effect of sericin on the hepatocyte transcriptome.

Research methods
Part 1 is a controlled trial comparing the novel serum-free medium and other media: C3A cells were cultured in our novel serum-free medium, HepatoZYME, complete medium (DMEM/F12 with 100 mL/L FBS), and DMEM/F12, then cell attachment, proliferation, and function as well as the biocompatibility of the media were assessed. Part 2 is a comparative study of serum-free media with or without 2 mg/mL sericin: The effect of sericin on C3A growth was assessed by cell viability and proliferation, the effect of sericin on C3A cell cycle distribution was determined by flow cytometry, and the effect of sericin on the C3A transcriptome was assessed by gene-chip array and RT-qPCR.

Research results
More C3A cells attached to the plate containing our serum-free medium than to those containing HepatoZYME and DMEM/F12 at 24 h post-seeding. Both the viability and proliferation rate of C3A cells in sericin-based serum-free medium were superior to those of cells in HepatoZYME and DMEM/F12. The content of albumin and urea in our serum-free medium was significantly higher than that in HepatoZYME and DMEM/F12 throughout the whole culture period, and was similar to that in complete medium at day 3, 4, and 5. In part 2, cell viability and proliferation were greater in the presence of 2 mg/mL sericin, as was the proportion of cells in S phase. Gene-chip array analysis indicated that the expression of *CCR6*, *EGFR*, and *FOS* were up-regulated by 2 mg/mL sericin, and RT-qPCR revealed that the expression of *CCR6*, *EGFR*, *FOS*, *AKT1*, *JNK1*, *NFkB1*, *MMP-9*, *MEK2*, *ERK1/2* and *C-MYC* was up-regulated by 2 mg/mL sericin.

Research conclusions
We developed a novel serum-free hepatocyte medium in this research and demonstrated that sericin probably enhances cell attachment through the

CCR6-Akt-JNK-NF-kB pathway and promotes cell proliferation through CCR6-mediated activation of the ERK1/2-MAPK pathway.

Research perspectives
In future studies, we will use the novel serum-free hepatocyte medium in large scale hepatocyte culture in the BALSS and assess the biocompatibility, immunogenicity and allergenicity in animal and clinical experiments. To clarify the mechanism of promotion of sericin on cell attachment and proliferation, we are going to study the protein level expression of CCR6-Akt-JNK-NF-kB pathway and ERK1/2-MAPK pathway components.

REFERENCES
1   **You S**, Zhu B, Liu H, Rong Y, Liu W, Zang H, Zhang A, Wan Z, Xin S. Safety of Human Hepatoma Cell-Line Constructing Bioartificial Liver Supporting System Treating Patients with Liver Failure. *Hepatogastroenterology* 2014; **61**: 933-936 [PMID: 26158144]
2   **Zhang Z**, Zhao YC, Cheng Y, Jian GD, Pan MX, Gao Y. Hybrid bioartificial liver support in cynomolgus monkeys with D-galactosamine-induced acute liver failure. *World J Gastroenterol* 2014; **20**: 17399-17406 [PMID: 25516651 DOI: 10.3748/wjg.v20.i46.17399]
3   **Lee JH**, Lee DH, Park JK, Kim SK, Kwon CH, Lee SK. Potentiality of immobilized pig hepatocyte spheroids in bioartificial liver system. *Transplant Proc* 2012; **44**: 1012-1014 [PMID: 22564612 DOI: 10.1016/j.transproceed.2012.03.010]
4   **Sheil AG**, Sun J, Mears DC, Waring M, Woodman K, Johnston B, Horvat M, Watson J, Koutalistras N, Wang L. Positive biochemical effects of a bioartificial liver support system (BALSS) in a porcine fulminant hepatic failure (FHF) model. *Int J Artif Organs* 1998; **21**: 43-48 [PMID: 9554825]
5   **Tsiaoussis J**, Newsome PN, Nelson LJ, Hayes PC, Plevris JN. Which hepatocyte will it be? Hepatocyte choice for bioartificial liver support systems. *Liver Transpl* 2001; **7**: 2-10 [PMID: 11150414 DOI: 10.1053/jlts.2001.20845]
6   **Thompson J**, Jones N, Al-Khafaji A, Malik S, Reich D, Munoz S, MacNicholas R, Hassanein T, Teperman L, Stein L, Duarte-Rojo A, Malik R, Adhami T, Asrani S, Shah N, Gaglio P, Duddempudi A, Borg B, Jalan R, Brown R, Patton H, Satoskar R, Rossi S, Parikh A, ElSharkawy A, Mantry P, Sher L, Wolf D, Hart M, Landis C, Wigg A, Habib S, McCaughan G, Colquhoun S, Henry A, Bedard P, Landeen L, Millis M, Ashley R, Frank W, Henry A, Stange J, Subramanian R; VTI-208 Study Group. Extracorporeal cellular therapy (ELAD) in severe alcoholic hepatitis: A multinational, prospective, controlled, randomized trial. *Liver Transpl* 2018; **24**: 380-393 [PMID: 29171941 DOI: 10.1002/lt.24986]
7   **van der Valk J**, Brunner D, De Smet K, Fex Svenningsen A, Honegger P, Knudsen LE, Lindl T, Noraberg J, Price A, Scarino ML, Gstraunthaler G. Optimization of chemically defined cell culture media--replacing fetal bovine serum in mammalian in vitro methods. *Toxicol In Vitro* 2010; **24**: 1053-1063 [PMID: 20362047 DOI: 10.1016/j.tiv.2010.03.016]
8   **Catania JR**, McGarrigle BP, Rittenhouse-Olson K, Olson JR. Induction of CYP2B and CYP2E1 in precision-cut rat liver slices cultured in defined medium. *Toxicol In Vitro* 2007; **21**: 109-115 [PMID: 17011741 DOI: 10.1016/j.tiv.2006.08.001]
9   **Oka K**, Ohya-Shimada W, Mizuno S, Nakamura T. Up-regulation of cyclin-E(1) via proline-mTOR pathway is responsible for HGF-mediated G(1)/S progression in the primary culture of rat hepatocytes. *Biochem Biophys Res Commun* 2013; **435**: 120-125 [PMID: 23618858 DOI: 10.1016/j.bbrc.2013.04.052]

10   **Yin M**, Yang H, Su X, Li Z, Yue Z, Zhang X, Sun D, Shi Y, Li D. Identification of EGF as an important regulator for promoting CYP3A4 expression in human embryonic stem cell-derived hepatocytes using TALEN-based gene targeting. *J Genet Genomics* 2014; **41**: 349-352 [PMID: 24976124 DOI: 10.1016/j.jgg.2014.03.008]

11   **Kimura M**, Moteki H, Ogihara M. Inhibitory effects of dexamethasone on hepatocyte growth factor-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. *J Pharmacol Sci* 2011; **115**: 390-398 [PMID: 21350311 DOI: 10.1254/jphs.10302FP]

12   **Ma R**, Zhang W, Tang K, Zhang H, Zhang Y, Li D, Li Y, Xu P, Luo S, Cai W, Ji T, Katirai F, Ye D, Huang B. Switch of glycolysis to gluconeogenesis by dexamethasone for treatment of hepatocarcinoma. *Nat Commun* 2013; **4**: 2508 [PMID: 24149070 DOI: 10.1038/ncomms3508]

13   **Zhaorigetu S**, Yanaka N, Sasaki M, Watanabe H, Kato N. Inhibitory effects of silk protein, sericin on UVB-induced acute damage and tumor promotion by reducing oxidative stress in the skin of hairless mouse. *J Photochem Photobiol B* 2003; **71**: 11-17 [PMID: 14705634 DOI: 10.1016/S1011-1344(03)00092-7]

14   **Kurioka A**, Kurioka F, Yamazaki M. Characterization of sericin powder prepared from citric acid-degraded sericin polypeptides of the silkworm, Bombyx Mori. *Biosci Biotechnol Biochem* 2004; **68**: 774-780 [PMID: 15118302 DOI: 10.1271/bbb.68.774]

15   **Sano M**, Tamada Y, Niwa K, Morita T, Yoshino G. Sulfated sericin is a novel anticoagulant influencing the blood coagulation cascade. *J Biomater Sci Polym Ed* 2009; **20**: 773-783 [PMID: 19323889 DOI: 10.1163/156856209X426853]

16   **Terada S**, Nishimura T, Sasaki M, Yamada H, Miki M. Sericin, a protein derived from silkworms, accelerates the proliferation of several mammalian cell lines including a hybridoma. *Cytotechnology* 2002; **40**: 3-12 [PMID: 19003099 DOI: 10.1023/a:1023993400608]

17   **Cao TT**, Zhang YQ. Viability and proliferation of L929, tumour and hybridoma cells in the culture media containing sericin protein as a supplement or serum substitute. *Appl Microbiol Biotechnol* 2015; **99**: 7219-7228 [PMID: 25895088 DOI: 10.1007/s00253-015-6576-3]

18   **Mandal BB**, Ghosh B, Kundu SC. Non-mulberry silk sericin/poly (vinyl alcohol) hydrogel matrices for potential biotechnological applications. *Int J Biol Macromol* 2011; **49**: 125-133 [PMID: 21549749 DOI: 10.1016/j.ijbiomac.2011.03.015]

19   **Mandal BB**, Priya AS, Kundu SC. Novel silk sericin/gelatin 3-D scaffolds and 2-D films: fabrication and characterization for potential tissue engineering applications. *Acta Biomater* 2009; **5**: 3007-3020 [PMID: 19398392 DOI: 10.1016/j.actbio.2009.03.026]

20   **Nayak S**, Dey S, Kundu SC. Silk sericin-alginate-chitosan microcapsules: hepatocytes encapsulation for enhanced cellular functions. *Int J Biol Macromol* 2014; **65**: 258-266 [PMID: 24486492 DOI: 10.1016/j.ijbiomac.2014.01.042]

21   **Miyamoto Y**, Teramoto N, Hayashi S, Enosawa S. An improvement in the attaching capability of cryopreserved human hepatocytes by a proteinaceous high molecule, sericin, in the serum-free solution. *Cell Transplant* 2010; **19**: 701-706 [PMID: 20525438 DOI: 10.3727/096368910X508799]

22   **Aramwit P**, Kanokpanont S, De-Eknamkul W, Kamei K, Srichana T. The effect of sericin with variable amino-acid content from different silk strains on the production of collagen and nitric oxide. *J Biomater Sci Polym Ed* 2009; **20**: 1295-1306 [PMID: 19520013 DOI: 10.1163/156856209X453006]

23   **Darzynkiewicz Z**, Halicka HD, Zhao H. Analysis of cellular DNA content by flow and laser scanning cytometry. *Adv Exp Med Biol* 2010; **676**: 137-147 [PMID: 20687474 DOI: 10.1007/978-1-4419-6199-0_9]

24   **Bowen WC**, Michalopoulos AW, Orr A, Ding MQ, Stolz DB, Michalopoulos GK. Development of a chemically defined medium and discovery of new mitogenic growth factors for mouse hepatocytes: mitogenic effects of FGF1/2 and PDGF. *PLoS One* 2014; **9**: e95487 [PMID: 24743506 DOI: 10.1371/journal.pone.0095487]

25   **Fernández-Fígares I**, Shannon AE, Wray-Cahen D, Caperna TJ. The role of insulin, glucagon, dexamethasone, and leptin in the regulation of ketogenesis and glycogen storage in primary cultures

of porcine hepatocytes prepared from 60 kg pigs. *Domest Anim Endocrinol* 2004; **27**: 125-140 [PMID: 15219932 DOI: 10.1016/j.domaniend.2004.02.003]

26   **Sahu N**, Pal S, Sapru S, Kundu J, Talukdar S, Singh NI, Yao J, Kundu SC. Non-Mulberry and Mulberry Silk Protein Sericins as Potential Media Supplement for Animal Cell Culture. *Biomed Res Int* 2016; **2016**: 7461041 [PMID: 27517047 DOI: 10.1155/2016/7461041]

27   **Dinescu S**, Gălățeanu B, Albu M, Lungu A, Radu E, Hermenean A, Costache M. Biocompatibility assessment of novel collagen-sericin scaffolds improved with hyaluronic Acid and chondroitin sulfate for cartilage regeneration. *Biomed Res Int* 2013; **2013**: 598056 [PMID: 24308001 DOI: 10.1155/2013/598056]

28   **Akturk O**, Tezcaner A, Bilgili H, Deveci MS, Gecit MR, Keskin D. Evaluation of sericin/collagen membranes as prospective wound dressing biomaterial. *J Biosci Bioeng* 2011; **112**: 279-288 [PMID: 21697006 DOI: 10.1016/j.jbiosc.2011.05.014]

29   **Aramwit P**, Kanokpanont S, De-Eknamkul W, Srichana T. Monitoring of inflammatory mediators induced by silk sericin. *J Biosci Bioeng* 2009; **107**: 556-561 [PMID: 19393558 DOI: 10.1016/j.jbiosc.2008.12.012]

30   **Glanemann M**, Knobeloch D, Ehnert S, Culmes M, Seeliger C, Seehofer D, Nussler AK. Hepatotropic growth factors protect hepatocytes during inflammation by upregulation of antioxidative systems. *World J Gastroenterol* 2011; **17**: 2199-2205 [PMID: 21633529 DOI: 10.3748/wjg.v17.i17.2199]

31   **Hou YT**, Ijima H, Matsumoto S, Kubo T, Takei T, Sakai S, Kawakami K. Effect of a hepatocyte growth factor/heparin-immobilized collagen system on albumin synthesis and spheroid formation by hepatocytes. *J Biosci Bioeng* 2010; **110**: 208-216 [PMID: 20547342 DOI: 10.1016/j.jbiosc.2010.01.016]

32   **Yang N**, Sun R, Liao X, Aa J, Wang G. UDP-glucuronosyltransferases (UGTs) and their related metabolic cross-talk with internal homeostasis: A systematic review of UGT isoforms for precision medicine. *Pharmacol Res* 2017; **121**: 169-183 [PMID: 28479371 DOI: 10.1016/j.phrs.2017.05.001]

33   **Shimada T**. Inhibition of Carcinogen-Activating Cytochrome P450 Enzymes by Xenobiotic Chemicals in Relation to Antimutagenicity and Anticarcinogenicity. *Toxicol Res* 2017; **33**: 79-96 [PMID: 28443179 DOI: 10.5487/TR.2017.33.2.079]

34   **Teo YL**, Saetaew M, Chanthawong S, Yap YS, Chan EC, Ho HK, Chan A. Effect of CYP3A4 inducer dexamethasone on hepatotoxicity of lapatinib: clinical and in vitro evidence. *Breast Cancer Res Treat* 2012; **133**: 703-711 [PMID: 22370628 DOI: 10.1007/s10549-012-1995-7]

35   **Maté ML**, Lifschitz A, Sallovitz J, Ballent M, Muscher AS, Wilkens MR, Schröder B, Lanusse C, Virkel G. Cytochrome P450 3A expression and function in liver and intestinal mucosa from dexamethasone-treated sheep. *J Vet Pharmacol Ther* 2012; **35**: 319-328 [PMID: 21906085 DOI: 10.1111/j.1365-2885.2011.01334.x]

36   **Terada S**, Sasaki M, Yanagihara K, Yamada H. Preparation of silk protein sericin as mitogenic factor for better mammalian cell culture. *J Biosci Bioeng* 2005; **100**: 667-671 [PMID: 16473778 DOI: 10.1263/jbb.100.667]

37   **Aramwit P**, Palapinyo S, Srichana T, Chottanapund S, Muangman P. Silk sericin ameliorates wound healing and its clinical efficacy in burn wounds. *Arch Dermatol Res* 2013; **305**: 585-594 [PMID: 23748948 DOI: 10.1007/s00403-013-1371-4]

38   **Nevzorova YA**, Tschaharganeh D, Gassler N, Geng Y, Weiskirchen R, Sicinski P, Trautwein C, Liedtke C. Aberrant cell cycle progression and endoreplication in regenerating livers of mice that lack a single E-type cyclin. *Gastroenterology* 2009; **137**: 691-703, 703.e1-703.e6 [PMID: 19445941 DOI: 10.1053/j.gastro.2009.05.003]

39   **Wallace AE**, Catalano RD, Anderson RA, Jabbour HN. Chemokine (C-C) motif ligand 20 is regulated by PGF(2α)-F-prostanoid receptor signalling in endometrial adenocarcinoma and promotes cell proliferation. *Mol Cell Endocrinol* 2011; **331**: 129-135 [PMID: 20816914 DOI: 10.1016/j.mce.2010.08.018]

40   **Nandi B**, Pai C, Huang Q, Prabhala RH, Munshi NC, Gold JS. CCR6, the sole receptor for the chemokine CCL20, promotes

spontaneous intestinal tumorigenesis. *PLoS One* 2014; **9**: e97566 [PMID: 24866282 DOI: 10.1371/journal.pone.0097566]

41   **Brand S**, Olszak T, Beigel F, Diebold J, Otte JM, Eichhorst ST, Göke B, Dambacher J. Cell differentiation dependent expressed CCR6 mediates ERK-1/2, SAPK/JNK, and Akt signaling resulting in proliferation and migration of colorectal cancer cells. *J Cell Biochem* 2006; **97**: 709-723 [PMID: 16215992 DOI: 10.1002/jcb.20672]

42   **Marsigliante S**, Vetrugno C, Muscella A. CCL20 induces migration and proliferation on breast epithelial cells. *J Cell Physiol* 2013; **228**: 1873-1883 [PMID: 23460117 DOI: 10.1002/jcp.24349]

43   **Fujii H**, Itoh Y, Yamaguchi K, Yamauchi N, Harano Y, Nakajima T, Minami M, Okanoue T. Chemokine CCL20 enhances the growth of HuH7 cells via phosphorylation of p44/42 MAPK in vitro. *Biochem Biophys Res Commun* 2004; **322**: 1052-1058 [PMID: 15336571 DOI: 10.1016/j.bbrc.2004.07.207]

44   **Gao M**, Zhan YQ, Yu M, Ge CH, Li CY, Zhang JH, Wang XH, Ge ZQ, Yang XM. Hepassocin activates the EGFR/ERK cascade and induces proliferation of L02 cells through the Src-dependent pathway. *Cell Signal* 2014; **26**: 2161-2166 [PMID: 24768768 DOI: 10.1016/j.cellsig.2014.04.013]

45   **Lanaya H**, Natarajan A, Komposch K, Li L, Amberg N, Chen L, Wculek SK, Hammer M, Zenz R, Peck-Radosavljevic M, Sieghart W, Trauner M, Wang H, Sibilia M. EGFR has a tumour-promoting role in liver macrophages during hepatocellular carcinoma formation. *Nat Cell Biol* 2014; **16**: 972-977 [PMID: 25173978 DOI: 10.1038/ncb3031]

46   **Biasin V**, Chwalek K, Wilhelm J, Best J, Marsh LM, Ghanim B, Klepetko W, Fink L, Schermuly RT, Weissmann N, Olschewski A, Kwapiszewska G. Endothelin-1 driven proliferation of pulmonary arterial smooth muscle cells is c-fos dependent. *Int J Biochem Cell Biol* 2014; **54**: 137-148 [PMID: 25016214 DOI: 10.1016/j.biocel.2014.06.020]

47   **Güller M**, Toualbi-Abed K, Legrand A, Michel L, Mauviel A, Bernuau D, Daniel F. c-Fos overexpression increases the proliferation of human hepatocytes by stabilizing nuclear Cyclin D1. *World J Gastroenterol* 2008; **14**: 6339-6346 [PMID: 19009649 DOI: 10.3748/wjg.14.6339]

48   **Long Y**, Wu Z, Yang X, Chen L, Han Z, Zhang Y, Liu J, Liu W, Liu X. MicroRNA-101 inhibits the proliferation and invasion of bladder cancer cells via targeting c-FOS. *Mol Med Rep* 2016; **14**: 2651-2656 [PMID: 27485165 DOI: 10.3892/mmr.2016.5534]

49   **Cheng X**, Yang Y, Fan Z, Yu L, Bai H, Zhou B, Wu X, Xu H, Fang M, Shen A, Chen Q, Xu Y. MKL1 potentiates lung cancer cell migration and invasion by epigenetically activating MMP9 transcription. *Oncogene* 2015; **34**: 5570-5581 [PMID: 25746000 DOI: 10.1038/onc.2015.14]

50   **Cortes-Reynosa P**, Robledo T, Macias-Silva M, Wu SV, Salazar EP. Src kinase regulates metalloproteinase-9 secretion induced by type IV collagen in MCF-7 human breast cancer cells. *Matrix Biol* 2008; **27**: 220-231 [PMID: 18061419 DOI: 10.1016/j.matbio.2007.11.003]

51   **Soto-Guzman A**, Navarro-Tito N, Castro-Sanchez L, Martinez-Orozco R, Salazar EP. Oleic acid promotes MMP-9 secretion and invasion in breast cancer cells. *Clin Exp Metastasis* 2010; **27**: 505-515 [PMID: 20617371 DOI: 10.1007/s10585-010-9340-1]

52   **Ebisuya M**, Kondoh K, Nishida E. The duration, magnitude and compartmentalization of ERK MAP kinase activity: mechanisms for providing signaling specificity. *J Cell Sci* 2005; **118**: 2997-3002 [PMID: 16014377 DOI: 10.1242/jcs.02505]

ORIGINAL ARTICLE

Basic Study

Total flavone of *Abelmoschus manihot* suppresses epithelial-mesenchymal transition *via* interfering transforming growth factor-β1 signaling in Crohn's disease intestinal fibrosis

Bo-Lin Yang, Ping Zhu, You-Ran Li, Min-Min Xu, Hao Wang, Li-Chao Qiao, Hai-Xia Xu, Hong-Jin Chen

Bo-Lin Yang, Ping Zhu, You-Ran Li, Min-Min Xu, Hao Wang, Li-Chao Qiao, Hai-Xia Xu, Hong-Jin Chen, Department of Colorectal Surgery, The Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing 210029, Jiangsu Province, China

ORCID number: Bo-Lin Yang (0000-0002-2474-4085); Ping Zhu (0000-0003-2583-7822); You-Ran Li (0000-0002-3179-6692); Min-Min Xu (0000-0001-6272-4643); Hao Wang (0000-0002-4058-2717); Li-Chao Qiao (0000-0292-2936); Hai-Xia Xu (0000-0002-2583-7822), Hong-Jin Chen (0000-0003-2222-3582).

Correspondence to: Hong-Jin Chen, MD, Chief Doctor, Department of Colorectal Surgery, The Affiliated Hospital of Nanjing University of Chinese Medicine, Hanzhong 155 Rd, Nanjing 210029, Jiangsu Province, China. 260789@njucm.edu.cn
Telephone: +86-13851887158

Abstract

AIM
To explore the role and mechanism of total flavone of *Abelmoschus manihot* (TFA) on epithelial-mesenchymal transition (EMT) progress of Crohn's disease (CD) intestinal fibrosis.

METHODS
First, CCK-8 assay was performed to assess TFA on the viability of intestinal epithelial (IEC-6) cells and select the optimal concentrations of TFA for our further studies. Then cell morphology, wound healing and transwell assays were performed to examine the effect of TFA on morphology, migration and invasion of IEC-6 cells treated with TGF-β1. In addition, immunofluorescence, real-time PCR analysis (qRT-PCR) and western blotting assays were carried out to detect the impact of TFA on EMT progress. Moreover, western blotting assay was performed to evaluate the function of TFA on the Smad and MAPK signaling pathways. Further, the role of co-treatment of TFA and si-Smad or MAPK inhibitors has been examined by qRT-PCR, western blotting, morphology, wound healing and

transwell assays.

RESULTS

In this study, TFA promoted transforming growth factor-β1 (TGF-β1)-induced (IEC-6) morphological change, migration and invasion, and increased the expression of epithelial markers and reduced the levels of mesenchymal markers, along with the inactivation of Smad and MAPK signaling pathways. Moreover, we revealed that si-Smad and MAPK inhibitors effectively attenuated TGF-β1-induced EMT in IEC-6 cells. Importantly, co-treatment of TFA and si-Smad or MAPK inhibitors had better inhibitory effects on TGF-β1-induced EMT in IEC-6 cells than either one of them.

CONCLUSION

These findings could provide new insight into the molecular mechanisms of TFA on TGF-β1-induced EMT in IEC-6 cells and TFA is expected to advance as a new therapy to treat CD intestinal fibrosis.

Key words: Crohn's disease; Intestinal fibrosis; Epithelial-to-mesenchymal transition; Total flavone of *Abelmoschus manihot*; Transforming growth factor-β1/Smad signaling; Transforming growth factor-β1/non-Smad signaling

Core tip: Regulating transforming growth factor-β (TGF-β) and its downstream signaling pathways, mediating the epithelial-mesenchymal transition (EMT) process and restoring the biological function of abnormally activated intestinal fibroblasts, may be an important way to seek drug therapy for Crohn's disease (CD) intestinal fibrosis. Total flavone of *Abelmoschus manihot* (TFA) can inhibit TGF-β1-induced morphological change, migration, invasion of rat intestinal epithelial cells, and promote induction of EMT partially by inhibiting TGF-β1-activated Smad and non-Smad signaling pathways. Therefore, TFA is expected to advance as a new therapy to treat CD intestinal fibrosis, and its continued advancement may open the door to a new class of treatment for CD intestinal fibrosis.

INTRODUCTION

Crohn's disease (CD) is a chronic relapsing inflammation of the gut, which causes significant impairment of quality of life with a rising incidence and prevalence during recent decades[1,2]. Although the clinical manifestations and pathologic progress of CD are different, fibrosis of intestinal organization and strictures induced by transmural inflammation will eventually cause intestinal obstruction, which is the characteristic clinical manifestation[3-5]. In addition, more than 1/3 of CD patients need at least one intestinal operation in their lives, while 70% of the CD patients with fibrosis strictures need partial resection of the intestinal tract within 10 years of disease progression, and 70%-90% patients will have a recurrence of anastomotic strictures and over 50% patients will form new strictures[6,7]. Moreover, a large number of clinical and experimental results have confirmed that the main drugs for treatment of CD, such as glucocorticoids, immune agents and biological agents, can effectively inhibit intestinal inflammation, but do not have positive activity in preventing the further progress of intestinal fibrosis[8,9]. Thus, there is still a lack of drugs that can effectively inhibit or reverse CD intestinal fibrosis.

The process of intestinal fibrosis in CD patients involves a variety of cells and multiple molecular signaling pathways[10,11]. Due to the continuous role of chronic intestinal inflammation, activated T and B cells will produce large amounts of pro-inflammatory cytokines and pro-fibrogenic factors, and induce fibroblast, epithelial cells, endothelial cells and stellate cells to migrate, proliferate, activate and differentiate into myofibroblasts, which finally results in excessive proliferation of myofibroblasts and excessive deposition of extracellular matrix (ECM), leading to the formation of intestinal fibrosis[12-14]. Studies have shown that even if inflammation of the intestinal tract is effectively controlled, the process of fibrosis will continue and eventually lead to intestinal stenosis[15]. Epithelial to mesenchymal transition (EMT) plays an important role in the activation of fibroblasts[16]. Epithelial cells will lose epithelial polarity and epithelial phenotype contacted with basement membrane and produce fibroblasts to repair tissue injury caused by trauma and inflammatory reactions through the EMT progress[17]. In physiological states, when the inflammatory reaction is relieved, the transformation process stops spontaneously. However, in the case of continuous activation of the inflammatory reaction, the EMT process will also continue to exist, and eventually cause organ fibrosis. Under pathophysiologic conditions, when the inflammatory reaction is relieved, the transformation process will stop spontaneously. However, in the case of continuous activation of inflammatory response, the EMT process will also exist continuously, and eventually cause organ fibrosis[18,19]. Nowadays, although the role and regulation mechanism of EMT in CD intestinal fibrosis has not been fully understood, the transforming growth factor-β (TGF-β)/Smad/MAPK signaling pathway has been confirmed to play an important role in regulating EMT in organs such as lung, liver, kidney and so on[20-22]. Therefore, studying the role of EMT in the formation of intestinal fibrosis based on the TGF-β/Smad/MAPK signaling pathway, may provide a new target for the treatment of CD intestinal fibrosis.
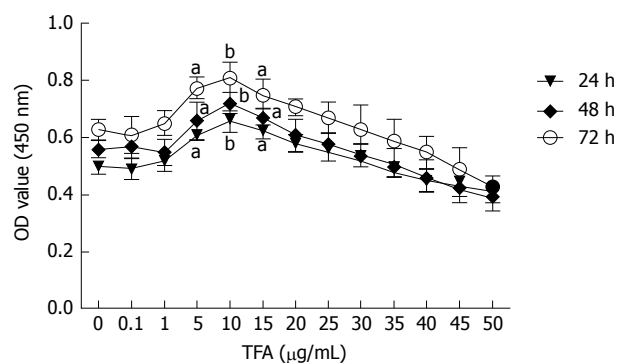
Figure 1 Effect of total flavone of *Abelmoschus manihot* at different concentrations on IEC-6 cell viability. IEC-6 cell viability after treatment with TFA at increasing concentrations was examined by CCK-8 at 24, 48 and 72 h. The results were expressed as the mean ± SD of three independent experiments, and each was performed in triplicate. [a]$P < 0.05$, [b]$P < 0.01$ compared with that of non-TFA treated group. TFA: Total flavone of *Abelmoschus manihot*.

Total flavone of *Abelmoschus manihot* L. Medic (TFA), as the main components of the water extract of traditional Chinese medicine *Abelmoschus manihot (L.)*, has been reported to play an important role in the improvement of renal inflammation, nephrotic syndrome, purpura nephritis, IgA nephropathy, membranous nephropathy and diabetic nephropathy (DN) effectively in the clinical trial[23-26]. Further, our previous research has shown that TFA could significantly inhibit the release of the intestinal inflammatory cytokines tumor necrosis factor (TNF)-α and interferon (IFN)-γ in a CD rat model induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS), improve the model animal survival rate, and effectively improve intestinal inflammation. Moreover, immunohistochemical staining showed that the expression of TGF-β, alpha-smooth muscle actin (α-SMA) and matrix metalloproteinase-2 (MMP-2) were obviously reduced after intervention of TFA. Further, Masson staining confirmed that collagen fibrils of the mucosa and lamina propria of the colon were markedly decreased, indicating that TFA had positive inhibitory effects on CD intestinal fibrosis, which may be related to the EMT mechanism mediated by TGF-β. Thus, we hypothesized that TFA could inhibit or reverse the CD intestinal fibrosis *via* regulation of EMT based on TGF-β and its downstream Smad and MAPK signaling pathways. Therefore, this study was designed to focus on the influence of EMT on CD intestinal fibrosis and to further explore the role and mechanism of TFA on the progress of CD intestinal fibrosis.

MATERIALS AND METHODS

Preparation of TFA
Abelmoschus Manihot L. Medic was collected from Jiangyan of Jiangsu province, China. TFA was extracted from the flowers of *Abelmoschus Manihot* by the Jiangsu Province Hospital of TCM, Nanjing, China. A total of 500 g of *Abelmoschus Manihot* flowers was immersed in 8000 mL 75% ethanol for 1 h. The mixture was refluxed

for 1 h at 90 ℃ and filtered by analytical filter paper. The extracts were evaporated by rotary evaporation under vacuum at 60 ℃[27]. For cell experiments, TFA was dissolved in dimethyl sulfoxide.

Cell culture
Rat intestinal epithelial (IEC-6) cells were obtained from the American Type Culture Collection (Manassas, VA, United States). Cells were cultured in Dulbecco's modified eagle's medium (DMEM, Gibco, United States) supplemented with 10% fetal bovine serum (FBS, Invitrogen, CA, United States), 2 mmol/L GlutaMAX-I (Invitrogen, CA, United States), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) in 5% CO_2 atmosphere at 37 ℃.

TFA working concentration
The concentration of TFA for further study was evaluated based on cell viability. Cell viability was determined by Cell Counting Kit-8 assay (CCK-8, Sigma Chemical Co, St Louis, MO, United States). Briefly, IEC-6 cells with a density of 1×10^4 cells/well in 100 μL of complete culture medium were seeded in 96-well plates. After culturing for 24 h, the medium was replaced with serum-free media or serum-free media containing TFA at concentrations ranging from 0.1 μg/mL to 50 μg/mL, and incubated in a humidified incubator at 37 ℃ for 24, 48 or 72 h. After incubation, 10 μL CCK-8 was added to each well for 2 h at 37 ℃. The optical density (OD) was recorded at 450 nm using a microplate reader (Dojindo Molecular Technology, Rockville, MD, United States).

Based on the results in Figure 1A, 5, 10, and 15 μg/mL TFA represented the optimal working concentrations and were used in our subsequent experiments.

Gene silencing with siRNAs
Cells were transfected with non-targeting negative control siRNA (Dharmacon, Lafayette, CO, United States) or Smad2/3 (Dharmacon, Lafayette, CO, United States) using LipoRNAiMax according to the manufacturer's protocol. The cells were maintained for 72 h and then subjected to protein extraction. The antisense and sense oligo template sequences for these siRNAs were: Smad2 siRNA antisense: AAGAGGAGTGCGCTTATATTACCTGTCTC, sense: AATAATATAAGCGCACTCCTCCCTGTCTC; Smad3 siRNA antisense: AATATTCCAGAAACCCCACCCCCTGTCTC, sense: AAGGGTGGGGTT TCTGGAATACCTGTCTC.

Wound healing assay
IEC-6 cells were seeded into 6-well plates at a density of 6×10^5 cells per well, and were cultured in fresh culture media to full confluence. After that, we created a wound using a plastic scraper. After being washed with PBS, the medium was replaced with 10 ng/mL TGF-β1[14] and TFA, and incubated at 37 ℃ for 48 h. The cell migration images were photographed at 0 and 48 h following scraping. Three to four different fields were visualized and photographed under a microscope (Nikon, Tokyo,

Japan).

Trans-well invasion assay

The cell invasion assay was performed using Trans-well chambers (8 μm pore-size, Corning, United States). Matrigel was purchased from BD Biosciences and stored at -20 ℃. After thawing at 4 ℃ overnight, the matrigel was diluted in serum-free medium, and 30 μL of the diluted matrigel were evenly inoculated into the upper chamber to form a gel at 37 ℃. Cells (1×10^5) suspended in 300 μL of serum-free medium were seeded into the upper compartments and treated with 10 ng/mL TGF-β1 and TFA for 48 h, and the lower compartments were filled with 600 μL of medium with 20% FBS. After incubation, the non-invasive cells were removed from the upper surface of the membrane by scrubbing. The cells that invaded to the lower surface of the membrane were fixed with 4% paraformaldehyde and stained in 10% crystal violet. Cells were counted under a microscope (Olympus, Tokyo, Japan).

Real-time PCR analysis (qRT-PCR)

IEC-6 cells were incubated in a 6-well plate with a density of 6×10^5 cell per well. When cells reached 60% confluence, 10 ng/mL TGF-β1 and TFA (0, 5, 10 and 15 μg/mL) were added to the plates and incubated for 48 h. Total RNA was extracted from the cultured cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions and reversed transcribed into cDNA using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, United States). The qRT-PCR reactions were performed using the Fast Start Universal SYBR Green Master (Rox) (Roche Applied Science) on a 7500 Real-time system (Applied Biosystems, United States) according to the manufacturer's protocols. Primer sequences used were designed as follows: E-cadherin forward, 5'-GAGGTCTACATTCCTGGGTG-3', E-cadherin reverse, 5'-TCTGTAGACATTTGAATCGG-3', ZO-1 forward, 5'-CCATCTTGGACCGATTGCTG-3', ZO-1 reverse, TAATGCCCGAGCTCCGATG-3', Vimentin forward, 5'-CCGACACTCCT ACAAGATTTAGA-3', Vimentin reverse, 5'-CAAAGATTTATTGAAGCAGAACC-3', N-cadherin forward, 5'-ATCCTACTGGACGGTTCG-3', N-cadherin reverse, 5'-TTGGCTAATGGCACTTGA-3', GAPDH forward, 5' -GGACCTGACCTGCCGTCTAG-3', GAPDH reverse, 5'-GTAGCCCAGGATG CCCTTGA-3'. GAPDH was used as an internal standard.

Western blotting assay

IEC-6 cells were plated into 6-well plates at a density of 3×10^5 cells/mL. When cells reached 60% confluence, 10 ng/mL TGF-β1 and TFA (0, 5, 10 and 15 μg/mL) were added to the plates and incubated for 48 h. Each concentration group was performed in triplicate. Protein lysates were prepared using RIPA lysis buffer. Lysates were then subjected to SDS-poly-acrylamide gel electrophoresis and transferred to PVDF membranes. After blocking with non-fat milk, blots were incubated overnight at 4 ℃ with the indicated antibodies. After incubation for 24 h, membranes were washed and incubated for 2 h at room temperature with corresponding secondary antibodies. For protein detection, membranes were developed with SuperSignal west femto maximum sensitivity substrate (Pierce, Rockford, IL, United States) and the Gel-Pro Analyzer 6.0 software was applied for image analysis. E-cadherin (1:500), ZO-1 (1:500), Vimentin (1:500), N-cadherin (1:500), p-ERK (1:500), ERK (1:500), p-p38 (1:500), p38 (1:500), p-JNK (1:500), JNK (1:500) and GAPDH (1:1000) antibodies were purchased from Sigma-Aldrich, and anti-mouse secondary antibodies were obtained from Proteintech (Chicago, IL, United States).

Immunofluorescence assay

IEC-6 cells were plated into 6-well plates at a density of 3×10^5 cells/mL. When cells reached 60% confluence, 10 ng/mL TGF-β1 and TFA (0, 5, 10 and 15 μg/mL) were added to the plates and incubated for 48 h. After that, IEC-6 cells were fixed with 4% paraformaldehyde. After blocking cells with 3% BSA for 2 h at room temperature, the cells were incubated with the anti-E-cadherin (1:50) antibody or anti-Vimentin (1:50) antibody at room temperature for 2 h. After washing three times with PBS, the second antibody conjugated with FITC was incubated on these cells for 1 h at room temperature. The nuclei were counterstained with DAPI for 5 min. Cells were imaged using a Nikon Eclipse TE2000-U fluorescence microscope.

Statistical analysis

Graph Pad Prism 5.0 statistical software was utilized to analyze the above experimental data. Measurement data were represented as X $\pm$ SD (n = 3); One-way analysis of variance was applied to compare differences between multiple groups. When only two groups were compared, Student's *t*-test was conducted. A value of $P < 0.05$ indicated that the difference was statistically significant.

RESULTS

Effect of TFA on IEC-6 cell viability

In order to observe the effect of TFA on the EMT progress of IEC-6 cells mediated by TGF-β1, a CCK-8 assay was performed to assess the effect of TFA on the viability of IEC-6 cells and to select the optimal concentrations of TFA for our further studies. IEC-6 cells were treated with increasing concentrations of TFA ranging from 0.1 to 50 μg/mL at different time points (24, 48 and 72 h). From the results of Figure 1, we found that TFA had the positive activities on viability of IEC-6 cells with the concentrations of 5, 10 and 15 μg/mL compared to the control group (TFA 0 μg/mL). Further, the TFA-treated group at 10 μg/mL displayed the maximum proliferative rate at 24, 48 and 72 h. Therefore, 5, 10 and 15 μg/mL TFA were chosen to be the optimal concentrations for our further studies.
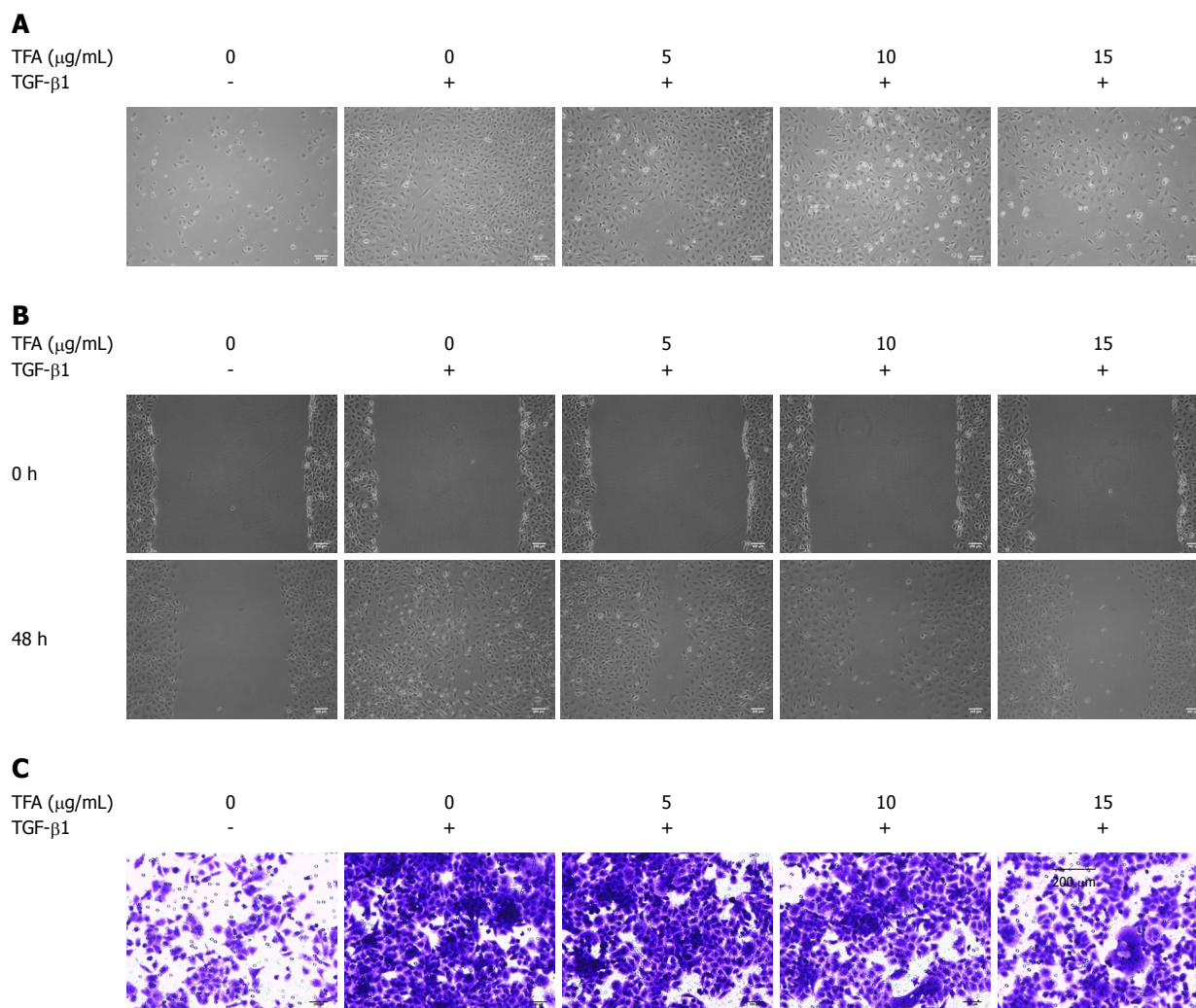
A

| TFA (μg/mL) | 0 | 0 | 5 | 10 | 15 |
| TGF-β1 | - | + | + | + | + |

B

| TFA (μg/mL) | 0 | 0 | 5 | 10 | 15 |
| TGF-β1 | - | + | + | + | + |

0 h

48 h

C

| TFA (μg/mL) | 0 | 0 | 5 | 10 | 15 |
| TGF-β1 | - | + | + | + | + |

Figure 2 Total flavone of *Abelmoschus manihot* inhibited transforming growth factor-β1 induced migration and invasion of IEC-6 cells. A: The inhibitory effect of TFA (5, 10 and 15 μg/mL) on TGF-β1-induced morphological changes at 48 h was observed by phase contrast microscopy; B: The representative image of the wound healing assay; C: The representative image of transwell assay. The tendency is concomitant with the wound healing assay. TGF-β1 induced the migration and invasion of IEC-6 cells, whereas TFA suppressed this phenomenon. TFA: Total flavone of *Abelmoschus manihot*; TGF: Transforming growth factor.

TFA inhibited TGF-β1 induced migration and invasion of IEC-6 cells

The EMT process is characterized by alteration of cell morphology, migration and invasion capacity, as well as epithelial and mesenchymal markers expression. In the morphology assay, 10 ng/mL TGF-β1 in the medium for 48 h led to significant morphological changes from cuboidal epithelial cells to fibroblast-like spindle-shaped cells when compared with the control group. Interestingly, TFA effectively suppressed TGF-β1-induced morphological changes of IEC-6 cells in a dose dependent manner (Figure 2A). Moreover, for wound healing assays, 10 ng/ml TGF-β1 could promote the migration of IEC-6 cells compared with the control group, while TFA obviously reduced the TGF-β1-induced migration of IEC-6 cells in a dose dependent manner (Figure 2B). In addition, we examined the effects of TFA on TGF-β1-induced invasion, and we found that 10 ng/mL TGF-β1 in the medium for 48 h induced IEC-6 cells across the membrane, and addition of 5, 10 and 15 μg/mL TFA

decreased the number of IEC-6 cells that passed though the Matrigel (Figure 2C). The results indicated that TFA could significantly suppress the morphological change, migration and invasion of IEC-6 cells induced by TGF-β1.

TFA inhibited TGF-β1-induced EMT

To examine the alterations of EMT markers, as shown in Figure 3A, the expression of epithelial marker E-cadherin was decreased along with the increasing expression of the mesenchymal marker Vimentin after treatment with 10 ng/mL TGF-β1. However, TFA effectively inhibited this induction, especially at higher concentrations. Besides, western blotting and qRT-PCR were carried out to examine the alterations of EMT markers, and we found that the protein levels of epithelial markers, including E-cadherin and ZO-1, were markedly increased by TFA treatment. On the contrary, TFA treatment obviously decreased the expression levels of mesenchymal proteins, including Vimentin and N-cadherin (Figure 3B). Likewise, similar changes were observed for mRNA

Figure 3 Total flavone of *Abelmoschus manihot* inhibited transforming growth factor-β1-induced epithelial-mesenchymal transition. A and B: The EMT-related label proteins were measured by immunocytochemistry (A) and western blotting (B) assays when IEC-6 cells were treated with TGF-β1 (10 ng/mL) alone or in combination with TFA (5, 10 and 15 μg/mL) for 48 h; C: The inhibitory effect of TFA (5, 10 and 15 μg/mL) on TGF-β1-induced EMT-related mRNAs was detected by qRT-PCR assay. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ compared with thef non-TFA treated group, [d]$P < 0.05$, [e]$P < 0.01$ compared with the TGF-β1 (10 ng/mL) treated group. EMT: Epithelial-mesenchymal transition; TFA: Total flavone of *Abelmoschus manihot*; TGF: Transforming growth factor.

expression of epithelial and mesenchymal markers in TFA-treated TGF-β1-induced IEC-6 cells (Figure 3C). Together, these results demonstrated that TFA could attenuate TGF-β1-induced EMT of IEC-6 cells.

TFA inhibited TGF-β1-induced activation of the Smad signaling pathway

The activation of phosphorylated Smad2/3 plays an important role in the TGF-β1-mediated EMT progress[28]. The status of the Smad pathways was determined in TGF-β1-treated IEC-6 cells following TFA treatment with the concentrations of 5, 10 and 15 μg/mL for 48 h, and the results have shown that TFA remarkably inhi-

bited the level of phosphorylated Smad 2/3 in a dose dependent manner (Figure 4A). Next, we examined the role of Smad in TGF-β1-mediated IEC-6 cells. In IEC-6 cells, western blotting analysis demonstrated that Smad2/3 expression was inhibited after transfection of si-Smad2/3 (Figure 4B). Moreover, our results revealed that si-Smad2/3 could diminish TGF-β1-triggered EMT in IEC-6 cells and inhibit the TGF-β1-elicited changes in the expression of EMT markers, as measured by western blotting and qRT-PCR assays (Figure 4B and C). Besides, si-Smad2/3 suppressed TGF-β1-mediated mensenchymal-like morphological changes in IEC-6 cells as shown in Figure 5A. Wound healing and trans-well
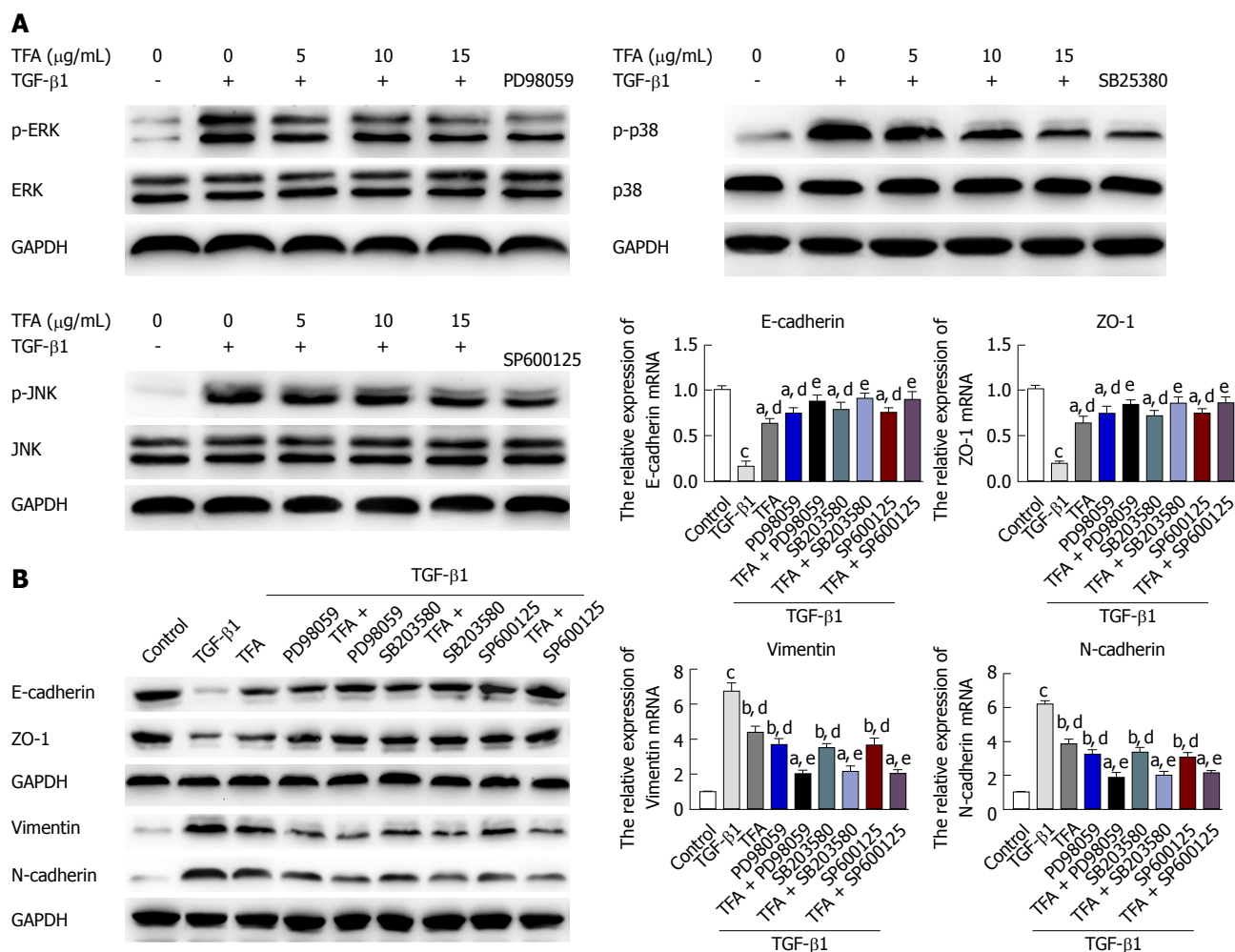
Figure 4 Total flavone of *Abelmoschus manihot* inhibited transforming growth factor-β1-induced activation of the Smad signaling pathway. A: The effects of TFA on TGF-β1-induced activation of Smad signaling were evaluated by western blotting; B: The expression of Smad signaling was examined by western blotting after transfection of si-Smad2/3; C and D: The effect of si-Smad or TFA combined with si-Smad2/3 on TGF-β1-induced activation of Smad signaling was evaluated by western blotting (C) and qRT-PCR (D) assays. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ compared with that of non-TFA treated group, [e]$P < 0.01$, [f]$P < 0.001$ compared with that of TGF-β1 (10 ng/mL) treated group. TFA: Total flavone of *Abelmoschus manihot*; TGF: Transforming growth factor.

assays also showed that si-Smad2/3 suppressed the migration and invasion capacities of IEC-6 cells triggered by TGF-β1 (Figure 5B and C). Furthermore, 15 μg/mL TFA combined with si-Smad2/3 could further restrain TGF-β1-mediated EMT progress in IEC-6 cells. These results suggested that TFA might reverse EMT induced by TGF-β1 *via* Smad inactivation in IEC-6 cells.

TFA inhibited TGF-β1-induced activation of the non-Smad signaling pathway

In addition to the Smad2/3 signaling pathway, TGF-β has been reported to activate other signaling molecules, such as MAPKs, which include extracellular signal-regulated kinase (ERK) 1/2, c-Jun NH2-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38 MAPK)[29]. To explore the involvement of non-Smad signaling in the anti TGF-β1-induced EMT activity of TFA, we tested whether TFA could inhibit the TGF-β1-induced

activation of p38, JNK and ERK1/2 by western blotting, and we found that TGF-β1 could significantly activate the expressions of p-p38, p-JNK and p-ERK1/2, while TFA at the concentrations of 5, 10 and 15 μg/mL obviously suppressed the TGF-β1-induced increase in p-p38, p-JNK and p-ERK1/2 levels in IEC-6 cells in a dose dependent manner (Figure 6A). Thus, in order to further explore the role of MAPKs in TGF-β1-treated IEC-6 cells, ERK1/2 inhibitor (PD98059, 5 μmol/L), p38 inhibitor (SB203580, 5 μmol/L) and JNK inhibitor (SP600125, 2 μmol/L) were used to treat IEC-6 cells mediated by TGF-β1. From the results in Figure 6A, we found that PD98059, SB203580 and SP600125 could significantly inhibit the activation of p-p38, p-JNK and p-ERK, respectively. Besides, PD98059, SB203580 and SP600125 could promote the changes in EMT markers by western blotting and qRT-PCR assays (Figure 6B and C). In addition, PD98059, SB203580 and SP600125 treatment inhibited TGF-β1-induced men-
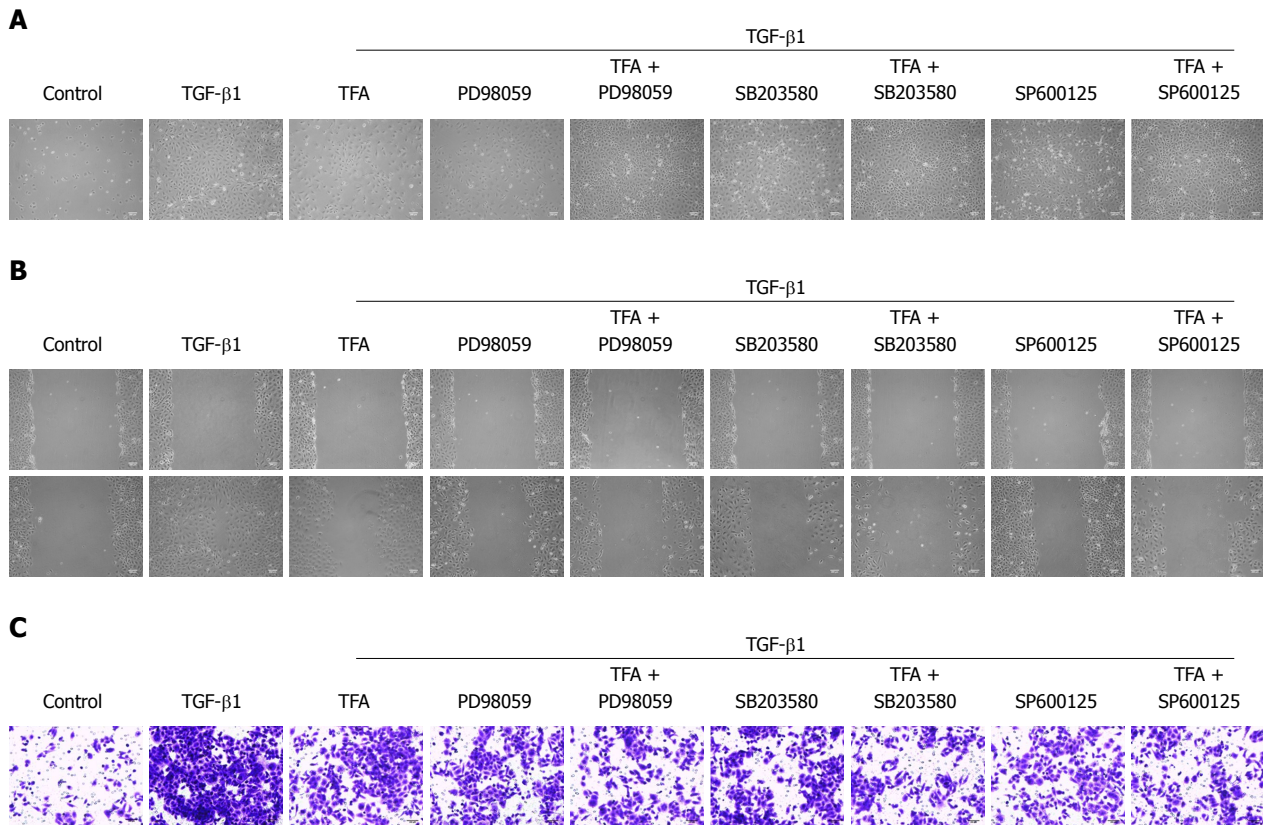
A

B

C

Figure 5 Total flavone of *Abelmoschus manihot* (TFA) combined with si-Smad2/3 had synergistic role in inhibiting transforming growth factor-β1-induced migration and invasion of IEC-6 cells. A: The inhibitory effect of si-Smad or TFA (15 μg/mL) combined with si-Smad2/3 on TGF-β1-induced morphological changes at 48 h was observed by phase contrast microscopy; B and C: The migration and invasion abilities of si-Smad or TFA (15 μg/mL) combined with si-Smad2/3 were examined by wound healing (B) and transwell (C) assays. TFA: Total flavone of *Abelmoschus manihot*; TGF: Transforming growth factor.a

senchymal-like morphological changes in IEC-6 cells, as shown in Figure 7A. Moreover, PD98059, SB203580 and SP600125 inhibited the migration and invasion of IEC-6 cells mediated by TGF-β1 *via* wound healing and transwell assays (Figure 7B and C). Importantly, co-treatment of TFA with PD98059, SB203580 or SP600125 had better inhibitory effects on TGF-β1-induced EMT in IEC-6 cells than either one of them. Taken together, these results indicated that TFA might exert its role in reversing TGF-β1-mediated EMT *via* non-Smad inactivation in IEC-6 cells.

DISCUSSION

CD intestinal fibrosis is characterized by activation of myofibroblasts, resulting in abnormal ECM deposition, which eventually leads to tissue stiffness and progressive intestinal dysfunction[30]. In addition to interstitial cells such as fibroblasts and smooth muscle cells, the differentiation of epithelial cells through EMT is a major source of intestinal fibrotic cells[18]. EMT refers to the transformation of epithelial cells with polar cells to the interstitial cells under a specific physiological and

pathological condition. The most important features of EMT are the loss of epithelial cell phenotype and the acquisition of interstitial properties, as reflected in the down-regulation of E-cadherin and ZO-1, resulting in loss of adhesion between cells or between cells and matrix, over-expression of N-cadherin and vimentin, leading to the acquisition of migration and invasion, and over-expression of EMT transcription factors[31,32]. Moreover, EMT plays a key role in tissue formation, organ fibrosis and so on. Through EMT, epithelial cells lose cell polarity and epithelial phenotypes, such as connection to the basement membrane, while acquiring interstitial cell phenotypes with the functions of fibroblasts and myofibroblasts[33-35]. Moreover, in the case of continuous activation of inflammatory response, the EMT process will also continuously exist and eventually cause organ fibrosis. Therefore, it will have a positive significance to search for a method for the treatment of CD intestinal fibrosis.

Our previous studies have found that TFA, a main component of the water extract of traditional Chinese medicine, could reduce the expression of TGF-β, α-SMA and MMP-2 and decrease collagen fibrils, which indi-

A

B

Figure 6 Total flavone of *Abelmoschus manihot* inhibited transforming growth factor-β1-induced activation of MAPK signaling pathway. A: The effect of TFA on TGF-β1-induced activation of MAPK signaling was evaluated by western blotting; B: The expression of MAPK signaling was examined by western blotting after transfection with MAPK inhibitors; B and C: The effects of MAPK inhibitors or TFA combined with MAPK inhibitors on TGF-β1-induced activation of Smad signaling were evaluated by western blotting (B) and qRT-PCR (C) assays. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ compared with that of non-TFA treated group, [d]$P < 0.05$, [e]$P < 0.01$ compared with that of TGF-β1 (10 ng/mL) treated group. TFA: Total flavone of *Abelmoschus manihot*; TGF: Transforming growth factor.

cated that TFA may inhibit or reverse EMT during CD intestinal fibrosis. Firstly, we used TGF-β1 to induce EMT of IEC-6 cells and chose the optimal concentrations of TFA to disrupt TGF-β1 activity in IEC-6 cells. From the results, we knew that TGF-β1 could obviously promote the progression of EMT, such as changing morphology from cuboidal epithelial cells to fibroblast-like spindle-shaped cells, and enhancing the abilities of migration and invasion. Despite this, TFA treatment could restrain the changes caused by TGF-β1 in a dose dependent manner.

Effective inhibition of CD intestinal inflammation does not play a positive role in preventing intestinal fibrosis and inhibiting the pro-fibrosis signaling pathway, resulting in persistence of the process of tissue fibrosis[36,37]. Therefore, it is necessary to further study how to block the fibrotic signaling pathway and promote ECM decomposition to inhibit or reverse the process of fibrosis. TGF-β is the strongest inducer, playing an important role in EMT[38]. The Smad signaling pathway induced by TGF-β can promote the expression of ECM and inhibit the transcriptional activity of matrix degradation

genes, resulting in ECM deposition in interstitial cells and leading to fibrosis[39]. Actually, in our experiments, we found that 10 ng/mL TGF-β1 could significantly inhibit the expression of p-Smad2/3, while TFA remarkably reversed the phenomenon in a dose dependent manner in TGF-β1-treated IEC-6 cells. Blocking TGF-β/Smad signaling has become a hot spot to inhibit or even reverse fibrosis. It has been reported that silencing Smad 2/3 could block Smad signaling and reduce collagen synthesis and proliferation of fibroblasts[40,41]. We silenced the expression of p-Smad2/3 and explored the role of Smad2/3 in TGF-β1-treated IEC-6 cells. We found that silencing Smad2/3 could effectively improve the EMT progression of IEC-6 cells induced by TGF-β1 no matter morphology, migration, invasion or EMT markers. Interestingly, when TFA was combined with si-Smad2/3, it had better inhibitory activities on EMT progression than either one of them alone, which suggested that TFA might exert its effects in reversing TGF-β1-mediated EMT *via* Smad inactivation in IEC-6 cells.

Recent researches have shown that the MAPK path-

A

B

C

Figure 7 Total flavone of *Abelmoschus manihot* combined with MAPK inhibitors had synergistic role in inhibiting transforming growth factor-β1 -induced migration and invasion of IEC-6 cells. A: The inhibitory effect of MAPK inhibitors or TFA (15 μg/mL) combined with MAPK inhibitors on TGF-β1-induced morphological changes at 48 h was observed by phase contrast microscopy; B and C: The migration and invasion abilities of MAPK inhibitors or TFA (15 μg/mL) combined with MAPK inhibitors were examined by wound healing (B) and transwell (C) assays. TFA: Total flavone of *Abelmoschus manihot*; TGF: Transforming growth factor.

way, including p38, JNK and ERK1/2, is closely related to the regulation of TGF-β signaling pathway and fibrosis[42]. TGF-β can not only carry out signal transduction through TGF-β/Smad, but can also activate p38, JNK and ERK signaling pathways, which indicates that regulation of MAPK signaling pathways can affect the TGF-β/Smad signal transduction pathway and play a role in inhibiting fibrosis[43,44]. In this experiment, we found that 10 ng/mL TGF-β1 could significantly inhibit the expression of p-p38, p-JNK and p-ERK1/2, while TFA obviously suppressed the levels in a dose dependent manner in TGF-β1-treated IEC-6 cells. In addition, we also investigated the role of MAPK in TGF-β1-mediated EMT of IEC-6 cells. We used an ERK1/2 inhibitor (PD98059), p38 inhibitor (SB203580) and JNK inhibitor (SP600125) to inhibit the expression of p-p38, p-JNK and p-ERK1/2 in IEC-6 cells, respectively. We found that PD98059, SB203580 and SP600125 treatment could have positive inhibitory effects on EMT progress, similar to the si-Smad. Moreover, co-treatment of TFA combined with PD98059, SB203580 or SP600125 had better positive activities on inhibiting EMT progress in TGF-β1-mediated IEC-6 cells than either one of them alone, indicating that TFA might exert its role in reversing EMT induced by TGF-β1 *via* MAPK inactivation in IEC-6 cells.

Stated thus, regulating TGF-β and its downstream Smad and MAPK signaling pathways, mediating the EMT process and restoring the biological function of abnormally activated intestinal fibroblasts, may be an important way to seek drug therapy for CD intestinal fibrosis. TFA is able to inhibit TGF-β1-induced morphological change, migration, invasion of IEC-6 cells. TFA promoted the induction of EMT partly by inhibiting TGF-β1-activated Smad signaling pathway and non-Smad signaling pathway. To conclude, TFA is expected to advance as a new therapy to treat CD intestinal fibrosis, and its continued advancement may open the door to a new class of treatment for CD intestinal fibrosis.

ARTICLE HIGHLIGHTS

Research background

Epithelial-mesenchymal transition (EMT) is a crucial process in Crohn's disease (CD) intestinal fibrosis. Total flavone of *Abelmoschus manihot* (TFA) has been found as an effective component to reduce CD intestinal fibrosis *in vivo*. However, the role and mechanism of TFA on EMT progress of CD intestinal fibrosis have not been understood yet.

Research motivation

EMT is a crucial process in CD intestinal fibrosis. TFA has been found as an effective component to reduce CD intestinal fibrosis *in vivo*. However, the role and mechanism of TFA on EMT progress of CD intestinal fibrosis have not been understood yet. In the present study, we performed CCK-8, morphology, wound

healing, transwell, qRT-PCR, western blotting and immunofluorescence assays to explore the role and the underlying mechanisms of TFA on CD intestinal fibrosis, and the results indicated that TFA was expected to advance as a new therapy to treat CD intestinal fibrosis.

Research objectives

To explore the role and mechanism of TFA on EMT progress of CD intestinal fibrosis.

Research methods

First, a CCK-8 assay was performed to assess the effect of TFA on the viability of IEC-6 cells and to select the optimal concentrations of TFA for our further studies. Then cell morphology, wound healing and transwell assays were performed to examine the effect of TFA on morphology, migration and invasion of IEC-6 cells treated with transforming growth factor-β1 (TGF-β1). In addition, immunofluorescence, qRT-PCR and western blotting assays were carried out to detect the impact of TFA on EMT progress. Moreover, western blotting assay was performed to evaluate the function of TFA on the Smad and MAPK signaling pathways. Further, the role of co-treatment of TFA and si-Smad or MAPK inhibitors was examined by qRT-PCR, western blotting, morphology, wound healing and transwell assays.

Research results

In this study, TFA promoted TGF-β1-induced IEC-6 cell morphological change, migration and invasion, and increased the expression of epithelial markers and reduced the levels of mesenchymal markers, along with the inactivation of Smad and MAPK signaling pathways. Moreover, we revealed that si-Smad and MAPK inhibitors effectively attenuated TGF-β1-induced EMT in TEC-6 cells. Importantly, co-treatment of TFA and si-Smad or MAPK inhibitors had better inhibitory effects on TGF-β1-induced EMT in IEC-6 cells than either one of them alone.

Research conclusions

These findings could provide new insight into the molecular mechanisms of TFA on TGF-β1-induced EMT in IEC-6 cells, and TFA is expected to advance as a new therapy to treat CD intestinal fibrosis.

Research perspectives

TFA promoted the induction of EMT partly by inhibiting TGF-β1-activated Smad signaling pathway and non-Smad signaling pathway. TFA is expected to advance as a new therapy to treat CD intestinal fibrosis, and its continued advancement may open the door to a new class of treatment for CD intestinal fibrosis.

REFERENCES

1   **Hirten RP,** Shah S, Sachar DB, Colombel JF. The Management of Intestinal Penetrating Crohn's Disease. *Inflamm Bowel Dis* 2018; **24**: 752-765 [PMID: 29528400 DOI: 10.1093/ibd/izx108]

2   **Stidham RW**, Wu J, Shi J, Lubman DM, Higgins PD. Serum Glycoproteome Profiles for Distinguishing Intestinal Fibrosis from Inflammation in Crohn's Disease. *PLoS One* 2017; **12**: e0170506 [PMID: 28114331 DOI: 10.1371/journal.pone.0170506]

3   **Wilkens R**, Hagemann-Madsen RH, Peters DA, Nielsen AH, Nørager CB, Glerup H, Krogh K. Validity of Contrast-enhanced Ultrasonography and Dynamic Contrast-enhanced MR Enterography in the Assessment of Transmural Activity and Fibrosis in Crohn's Disease. *J Crohns Colitis* 2018; **12**: 48-56 [PMID: 28981627 DOI: 10.1093/ecco-jcc/jjx111]

4   **Rogler G**, Hausmann M. Factors Promoting Development of Fibrosis in Crohn's Disease. *Front Med* (Lausanne) 2017; **4**: 96 [PMID: 28736729 DOI: 10.3389/fmed.2017.00096]

5   **Hui CK**, Hui NK. Collagenous colitis presenting with skip lesions mimicking Crohn's disease and complicated by intestinal obstruction. *J Dig Dis* 2017; **18**: 487-491 [PMID: 28682012 DOI: 10.1111/1751-2980.12507]

6   **Ma C**, Moran GW, Benchimol EI, Targownik LE, Heitman SJ, Hubbard JN, Seow CH, Novak KL, Ghosh S, Panaccione R, Kaplan GG. Surgical Rates for Crohn's Disease are Decreasing: A Population-Based Time Trend Analysis and Validation Study. *Am J Gastroenterol* 2017; **112**: 1840-1848 [PMID: 29087396 DOI: 10.1038/ajg.2017.394]

7   **Ha FJ**, Thong L, Khalil H. Quality of Life after Intestinal Resection in Patients with Crohn Disease: A Systematic Review. *Dig Surg* 2017; **34**: 355-363 [PMID: 28099962 DOI: 10.1159/000453590]

8   **Sobrado CW**, Leal RF, Sobrado LF. THERAPIES FOR CROHN'S DISEASE: a clinical update. *Arq Gastroenterol* 2016; **53**: 206-211 [PMID: 27438429 DOI: 10.1590/S0004-28032016000300016]

9   **Deepak P**, Loftus EV Jr. Ustekinumab in treatment of Crohn's disease: design, development, and potential place in therapy. *Drug Des Devel Ther* 2016; **10**: 3685-3698 [PMID: 27956825 DOI: 10.2147/DDDT.S102141]

10   **Wang X**, Lu Y, Wu L, Zhao C, Song C, Yu S, Zhao B, Zhao T, Liu H, Dou C, Zhang Y, Wu H. Moxibustion Inhibits the ERK Signaling Pathway and Intestinal Fibrosis in Rats with Crohn's Disease. *Evid Based Complement Alternat Med* 2013; **2013**: 198282 [PMID: 23970928 DOI: 10.1155/2013/198282]

11   **Abreu MT**, Taylor KD, Lin YC, Hang T, Gaiennie J, Landers CJ, Vasiliauskas EA, Kam LY, Rojany M, Papadakis KA, Rotter JI, Targan SR, Yang H. Mutations in NOD2 are associated with fibrostenosing disease in patients with Crohn's disease. *Gastroenterology* 2002; **123**: 679-688 [PMID: 12198692 DOI: 10.1053/gast.2002.35393]

12   **Hassan-Zahraee M**, Banerjee A, Cheng JB, Zhang W, Ahmad A, Page K, von Schack D, Zhang B, Martin SW, Nayak S, Reddy P, Xi L, Neubert H, Fernandez Ocana M, Gorelick K, Clare R, Vincent M, Cataldi F, Hung K. Anti-MAdCAM Antibody Increases ß7+ T Cells and CCR9 Gene Expression in the Peripheral Blood of Patients With Crohn's Disease. *J Crohns Colitis* 2018; **12**: 77-86 [PMID: 28961803 DOI: 10.1093/ecco-jcc/jjx121]

13   **Zheng Y**, Ge W, Ma Y, Xie G, Wang W, Han L, Bian B, Li L, Shen L. miR-155 Regulates IL-10-Producing CD24^{hi}CD27B^{+} Cells and Impairs Their Function in Patients with Crohn's Disease. *Front Immunol* 2017; **8**: 914 [PMID: 28824639 DOI: 10.3389/fimmu.2017.00914]

14   **Mortensen JH**, Godskesen LE, Jensen MD, Van Haaften WT, Klinge LG, Olinga P, Dijkstra G, Kjeldsen J, Karsdal MA, Bay-Jensen AC, Krag A. Fragments of Citrullinated and MMP-degraded Vimentin and MMP-degraded Type III Collagen Are Novel Serological Biomarkers to Differentiate Crohn's Disease from Ulcerative Colitis. *J Crohns Colitis* 2015; **9**: 863-872 [PMID: 26188349 DOI: 10.1093/ecco-jcc/jjv123]

15   **Kurahara LH**, Hiraishi K, Sumiyoshi M, Doi M, Hu Y, Aoyagi K, Jian Y, Inoue R. Significant contribution of TRPC6 channel-mediated Ca^{2+} influx to the pathogenesis of Crohn's disease fibrotic stenosis. *J Smooth Muscle Res* 2016; **52**: 78-92 [PMID: 27818466 DOI: 10.1540/jsmr.52.78]

16   **Jiang H**, Shen J, Ran Z. Epithelial-mesenchymal transition in Crohn's disease. *Mucosal Immunol* 2018; **11**: 294-303 [PMID: 29346350 DOI: 10.1038/mi.2017.107]

17   **Scharl M**, Frei P, Frei SM, Biedermann L, Weber A, Rogler G. Epithelial-to-mesenchymal transition in a fistula-associated anal adenocarcinoma in a patient with long-standing Crohn's disease. *Eur J Gastroenterol Hepatol* 2014; **26**: 114-118 [PMID: 24284372 DOI: 10.1097/MEG.0b013e32836371a2]

18   **Scharl M**, Huber N, Lang S, Fürst A, Jehle E, Rogler G. Hallmarks of epithelial to mesenchymal transition are detectable in Crohn's disease associated intestinal fibrosis. *Clin Transl Med* 2015; **4**: 1 [PMID: 25852817 DOI: 10.1186/s40169-015-0046-5]

19   **Chen Y**, Xiao Y, Ge W, Zhou K, Wen J, Yan W, Wang Y, Wang B, Qu C, Wu J, Xu L, Cai W. miR-200b inhibits TGF-β1-induced epithelial-mesenchymal transition and promotes growth of intestinal epithelial cells. *Cell Death Dis* 2013; **4**: e541 [PMID: 23492772 DOI: 10.1038/cddis.2013.22]

20   **Liu TJ**, Guo JL, Wang HK, Xu X. Semaphorin-7A contributes to growth, migration and invasion of oral tongue squamous cell carcinoma through TGF-β-mediated EMT signaling pathway. *Eur*

Rev Med Pharmacol Sci 2018; **22**: 1035-1043 [PMID: 29509252]

21 **Sommerova L**, Ondrouskova E, Vojtesek B, Hrstka R. Suppression of AGR2 in a TGF-β-induced Smad regulatory pathway mediates epithelial-mesenchymal transition. *BMC Cancer* 2017; **17**: 546 [PMID: 28810836 DOI: 10.1186/s12885-017-3537-5]

22 **Sun S**, Xie F, Zhang Q, Cui Z, Cheng X, Zhong F, He K, Zhou J. Advanced oxidation protein products induce hepatocyte epithelial-mesenchymal transition via a ROS-dependent, TGF-β/Smad signaling pathway. *Cell Biol Int* 2017; **41**: 842-853 [PMID: 28500745 DOI: 10.1002/cbin.10792]

23 **Liu Z**, Liu S, Zhou L, Gao X, Ju W, Tan H, Yang C. Effects of HuangKui capsules on glibenclamide pharmacokinetics in rats. *J Ethnopharmacol* 2012; **139**: 1-5 [PMID: 21527329 DOI: 10.1016/j.jep.2011.03.043]

24 **Tu Y**, Sun W, Wan YG, Che XY, Pu HP, Yin XJ, Chen HL, Meng XJ, Huang YR, Shi XM. Huangkui capsule, an extract from Abelmoschus manihot (L.) medic, ameliorates adriamycin-induced renal inflammation and glomerular injury via inhibiting p38MAPK signaling pathway activity in rats. *J Ethnopharmacol* 2013; **147**: 311-320 [PMID: 23518420 DOI: 10.1016/j.jep.2013.03.006]

25 **Ge J**, Miao JJ, Sun XY, Yu JY. Huangkui capsule, an extract from Abelmoschus manihot (L.) medic, improves diabetic nephropathy via activating peroxisome proliferator-activated receptor (PPAR)-α/γ and attenuating endoplasmic reticulum stress in rats. *J Ethnopharmacol* 2016; **189**: 238-249 [PMID: 27224243 DOI: 10.1016/j.jep.2016.05.033]

26 **Liu S**, Ye L, Tao J, Ge C, Huang L, Yu J. Total flavones of Abelmoschus manihot improve diabetic nephropathy by inhibiting the iRhom2/TACE signalling pathway activity in rats. *Pharm Biol* 2017; **56**: 1-11 [PMID: 29221422 DOI: 10.1080/13880209.2017.1412467]

27 **Guo J**, Xue C, Duan JA, Qian D, Tang Y, You Y. Anticonvulsant, antidepressant-like activity of Abelmoschus manihot ethanol extract and its potential active components in vivo. *Phytomedicine* 2011; **18**: 1250-1254 [PMID: 21784623 DOI: 10.1016/j.phymed.2011.06.012]

28 **Chen Q**, Yang W, Wang X, Li X, Qi S, Zhang Y, Gao MQ. TGF-β1 Induces EMT in Bovine Mammary Epithelial Cells Through the TGFβ1/Smad Signaling Pathway. *Cell Physiol Biochem* 2017; **43**: 82-93 [PMID: 28848180 DOI: 10.1159/000480321]

29 **Ling G**, Ji Q, Ye W, Ma D, Wang Y. Epithelial-mesenchymal transition regulated by p38/MAPK signaling pathways participates in vasculogenic mimicry formation in SHG44 cells transfected with TGF-β cDNA loaded lentivirus in vitro and in vivo. *Int J Oncol* 2016; **49**: 2387-2398 [PMID: 27748800 DOI: 10.3892/ijo.2016.3724]

30 **Cayci M**, Bostanci EB, Turhan N, Karaman K, Dalgic T, Ozer I, Ercan M, Ulas M, Akoglu M. The analysis of clinico-pathologic characteristics in patients who underwent surgery due to stricturing and non-perineal fistulizing forms of Crohn's disease: a retrospective cohort study. *Int J Surg* 2015; **15**: 49-54 [PMID: 25647540 DOI: 10.1016/j.ijsu.2015.01.029]

31 **Conway J**, Al-Zahrani KN, Pryce BR, Abou-Hamad J, Sabourin LA. Transforming growth factor β-induced epithelial to mesenchymal transition requires the Ste20-like kinase SLK independently of its catalytic activity. *Oncotarget* 2017; **8**: 98745-98756 [PMID: 29228724 DOI: 10.18632/oncotarget.21928]

32 **Ma F**, Li W, Liu C, Li W, Yu H, Lei B, Ren Y, Li Z, Pang D, Qian C. MiR-23a promotes TGF-β1-induced EMT and tumor metastasis in breast cancer cells by directly targeting CDH1 and activating Wnt/β-catenin signaling. *Oncotarget* 2017; **8**: 69538-69550 [PMID: 29050223]

33 **You J**, Li M, Tan Y, Cao L, Gu Q, Yang H, Hu C. Snail1-expressing cancer-associated fibroblasts induce lung cancer cell epithelial-mesenchymal transition through miR-33b. *Oncotarget* 2017; **8**: 114769-114786 [PMID: 29383119 DOI: 10.18632/oncotarget.23082]

34 **Ke X**, Yang D, Liang J, Wang X, Wu S, Wang X, Hu C. Human Endothelial Progenitor Cell-Derived Exosomes Increase Proliferation and Angiogenesis in Cardiac Fibroblasts by Promoting the Mesenchymal-Endothelial Transition and Reducing High Mobility Group Box 1 Protein B1 Expression. *DNA Cell Biol* 2017; **36**: 1018-1028 [PMID: 28920705 DOI: 10.1089/dna.2017.3836]

35 **Guan R**, Wang X, Zhao X, Song N, Zhu J, Wang J, Wang J, Xia C, Chen Y, Zhu D, Shen L. Emodin ameliorates bleomycin-induced pulmonary fibrosis in rats by suppressing epithelial-mesenchymal transition and fibroblast activation. *Sci Rep* 2016; **6**: 35696 [PMID: 27774992 DOI: 10.1038/srep35696]

36 **Boland BS**, Vermeire S. Janus Kinase Antagonists and Other Novel Small Molecules for the Treatment of Crohn's Disease. *Gastroenterol Clin North Am* 2017; **46**: 627-644 [PMID: 28838419 DOI: 10.1016/j.gtc.2017.05.015]

37 **Ramos GP**, Faubion WA, Papadakis KA. Targeting Specific Immunologic Pathways in Crohn's Disease. *Gastroenterol Clin North Am* 2017; **46**: 577-588 [PMID: 28838416 DOI: 10.1016/j.gtc.2017.05.009]

38 **Chen H**, Chen Q, Jiang CM, Shi GY, Sui BW, Zhang W, Yang LZ, Li ZY, Liu L, Su YM, Zhao WC, Sun HQ, Li ZZ, Fu Z. Triptolide suppresses paraquat induced idiopathic pulmonary fibrosis by inhibiting TGFB1-dependent epithelial mesenchymal transition. *Toxicol Lett* 2018; **284**: 1-9 [PMID: 29195901 DOI: 10.1016/j.toxlet.2017.11.030]

39 **Perumal N**, Perumal M, Halagowder D, Sivasithamparam N. Morin attenuates diethylnitrosamine-induced rat liver fibrosis and hepatic stellate cell activation by co-ordinated regulation of Hippo/Yap and TGF-β1/Smad signaling. *Biochimie* 2017; **140**: 10-19 [PMID: 28552397 DOI: 10.1016/j.biochi.2017.05.017]

40 **Luong VH**, Chino T, Oyama N, Matsushita T, Sasaki Y, Ogura D, Niwa SI, Biswas T, Hamasaki A, Fujita M, Okamoto Y, Otsuka M, Ihn H, Hasegawa M. Blockade of TGF-β/Smad signaling by the small compound HPH-15 ameliorates experimental skin fibrosis. *Arthritis Res Ther* 2018; **20**: 46 [PMID: 29544542 DOI: 10.1186/s13075-018-1534-y]

41 **Ma YL**, Chen F, Yang SX, Chen BP, Shi J. MicroRNA-21 promotes the progression of peritoneal fibrosis through the activation of the TGF-β/Smad signaling pathway: An in vitro and in vivo study. *Int J Mol Med* 2018; **41**: 1030-1038 [PMID: 29207016]

42 **Cao Y**, Liu Y, Ping F, Yi L, Zeng Z, Li Y. miR-200b/c attenuates lipopolysaccharide-induced early pulmonary fibrosis by targeting ZEB1/2 via p38 MAPK and TGF-β/smad3 signaling pathways. *Lab Invest* 2018; **98**: 339-359 [PMID: 29200203 DOI: 10.1038/labinvest.2017.123]

43 **Bansal T**, Chatterjee E, Singh J, Ray A, Kundu B, Thankamani V, Sengupta S, Sarkar S. Arjunolic acid, a peroxisome proliferator-activated receptor α agonist, regresses cardiac fibrosis by inhibiting non-canonical TGF-β signaling. *J Biol Chem* 2017; **292**: 16440-16462 [PMID: 28821620 DOI: 10.1074/jbc.M117.788299]

44 **Che X**, Wang Q, Xie Y, Xu W, Shao X, Mou S, Ni Z. Astragaloside IV suppresses transforming growth factor-β1 induced fibrosis of cultured mouse renal fibroblasts via inhibition of the MAPK and NF-κB signaling pathways. *Biochem Biophys Res Commun* 2015; **464**: 1260-1266 [PMID: 26220342 DOI: 10.1016/j.bbrc.2015.07.116]

Basic Study

Identification of a five-long non-coding RNA signature to improve the prognosis prediction for patients with hepatocellular carcinoma

Qiu-Jie Zhao, Jiao Zhang, Lin Xu, Fang-Feng Liu

Qiu-Jie Zhao, Jiao Zhang, Lin Xu, Department of Gastroenterology, Shandong Provincial Hospital affiliated to Shandong University, Jinan 250021, Shandong Province, China

Fang-Feng Liu, Department of Hepatobiliary Surgery, Shandong Provincial Hospital affiliated to Shandong University, Jinan 250021, Shandong Province, China

ORCID number: Qiu-Jie Zhao (0000-0003-4053-8406); Jiao Zhang (0000-0002-9178-1003); Lin Xu (0000-0003-0253-608X); Fang-Feng Liu (0000-0002-3745-5012).

Institutional review board statement: This studied mined the TCGA database and doesn't involve any experiments with animals or human beings. Because the TCGA data are a community resource project, additional ethical approval was not acquired.

Conflict-of-interest statement: The authors declare that there is no conflict of interest related to this study.

Data sharing statement: The datasets supporting the conclusions of this article are included within the article.

Correspondence to: Qiu-Jie Zhao, PhD, Chief Doctor, Department of Gastroenterology, Shandong Provincial Hospital affiliated to Shandong University, No. 324, Jingwuwei 7th Road, Jinan 250021, Shandong Province, China. zhaoqiujiesdu@126.com
Telephone: +86-531-68772952

Abstract

AIM

To construct a long non-coding RNA (lncRNA) signature for predicting hepatocellular carcinoma (HCC) prognosis with high efficiency.

METHODS

Differentially expressed lncRNAs (DELs) between HCC specimens and peritumor liver specimens were identified using the edgeR package to analyze The Cancer Genome Atlas (TCGA) LIHC dataset. Univariate Cox proportional hazards regression was performed to obtain the DELs significantly associated with overall survival (OS) in a training set. These OS-related DELs were further analyzed using a stepwise multivariate Cox regression model. Those lncRNAs fitted in the multivariate Cox regression model and independently associated with overall survival were chosen to build a prognostic risk formula. The prognostic value of

this formula was then validated in the test group and the entire cohort and further compared with two previously identified prognostic signatures for HCC. Gene ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses were performed to explore the potential biological functions of the lncRNAs in the signature.

RESULTS
Based on lncRNA expression profiling of 370 HCC patients from the TCGA database, we constructed a 5-lncRNA signature (AC015908.3, AC091057.3, TMCC1-AS1, DCST1-AS1 and FOXD2-AS1) that was significantly associated with prognosis. HCC patients with high-risk scores based on the expression of the 5 lncRNAs had significantly shorter survival times compared to patients with low-risk scores in both the training and test groups. Multivariate Cox regression analysis demonstrated that the prognostic value of the 5 lncRNAs was independent of clinicopathological parameters. A comparison study involving two previously identified prognostic signatures for HCC demonstrated that this 5-lncRNA signature showed improved prognostic power compared with the other two signatures. Functional enrichment analysis indicated that the 5 lncRNAs were potentially involved in metabolic processes, fibrinolysis and complement activation.

CONCLUSION
Our present study constructed a 5-lncRNA signature that improves survival prediction and can be used as a prognostic biomarker for HCC patients.

Key words: Long non-coding RNA; Hepatocellular carcinoma; Prognosis; Survival prediction; Prognostic biomarker

Core tip: In the present study, we developed a 5-long non-coding RNA (lncRNA) signature for predicting the prognosis of hepatocellular carcinoma (HCC) patients based on The Cancer Genome Atlas database. The signature was reproducible and robust in another independent large-scale HCC cohort, supporting its utility and effectiveness. In addition, the prognostic value of the 5-lncRNA signature was independent of clinicopathological variables. When compared with two previously identified signatures for HCC survival prediction, this 5-lncRNA signature showed superior prognostic power. Our study indicates that the 5-lncRNA signature could improve survival prediction and could be used as a prognostic biomarker for HCC patients.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most commonly diagnosed cancer in the world[1]. According to previous epidemiologic studies, the incidence of HCC varies strikingly worldwide and is particularly high in eastern Asian countries, including China, and sub-Saharan Africa[2,3]. The 5-year overall survival rate for HCC is lower than 20%[4], and the ratio of its mortality to morbidity is 0.95[1]. Because of its poor prognosis, HCC ranks as the second leading cause of cancer-related deaths worldwide[1]. An estimated 782500 new liver cancer cases and 745500 deaths occurred worldwide in 2012, among which 50% occurred in China[3]. There are multiple risk factors related to HCC, including hepatitis B or C viral infection, chronic alcohol abuse, nonalcoholic fatty liver disease and smoking[5,6]. Although treatment for HCC, including surgical resection, has improved over the past decades, the overall survival rate for this disease remains devastatingly high due to its high recurrence rate (50%-70% at 5 years)[7-9]. Because HCC is a heterogeneous disease with substantially variable clinical outcomes, the search for effective biomarkers to predict recurrence and prognosis is indispensable. To date, no widely accepted molecular biomarkers for HCC aggressiveness are available. In the past 40 years, serum alpha fetoprotein (AFP) levels have been utilized for the diagnosis of HCC and for predicting its response to therapy. However, AFP levels can be influenced by tumor size and cancer stage, and they are not reliable in clinical applications[10]. In addition, the American Association for the Study of Liver Diseases concluded that the use of AFP levels lacks sufficient sensitivity and specificity to effectively monitor or diagnose HCC[11].

With the development of high-throughput sequencing technologies, it has become easy to acquire whole genome profiles for specific cancers and develop more reliable prognostic signatures. Long non-coding RNAs (lncRNAs) are mRNA-like transcripts of more than 200 nucleotides (nt) with little or no protein-coding capacity[12,13]. In the past, they were previously thought to be redundant segments of the genome, but in recent decades, emerging studies have indicated the importance of lncRNAs in cellular physiological and pathological processes[14,15]. Increasing evidence suggests that dysregulated lncRNAs are associated with various human diseases, particularly the initiation and progression of various human cancers[16,17]. Prognostic lncRNA signatures have been examined in many cancer types, including renal cancer, glioblastoma, colorectal cancer, lymphoma, and others[18-21]. For HCC, most of the published gene signatures associated with prognosis have focused on mRNAs and microRNAs[22-25]. To the

Table 1 Clinicopathological parameters of hepatocellular carcinoma patients in each cohort				
Variables		Training group (*n* = 184)	Test group (*n* = 186)	Entire group (*n* = 370)
Age, yr	< 60	84	85	169
	⩾ 60	100	101	201
Sex	Male	129	120	24
	Female	55	66	121
Weight, kg	< 70	92	85	177
	⩾ 70	78	89	167
	NA	14	12	26
Child-Pugh grade	A	109	107	216
	B	9	12	21
	C	0	1	1
	NA	66	66	132
Fibrosis ishak score	0	32	42	74
	1-5	38	31	69
	6	36	33	69
	NA	78	80	158
Vascular tumor invasion	None	100	106	206
	Micro	45	46	91
	Macro	8	9	17
	NA	31	25	56
Serum FAP level, ng/mL	< 100	102	90	192
	⩾ 100	37	48	85
	NA	45	48	93
Tumor grade	1	37	18	55
	2	82	95	177
	3 + 4	61	72	133
	NA	4	1	5
Pathologic stage	I	92	79	171
	II	43	42	85
	III + IV	37	53	90
	NA	12	12	24

best of our knowledge, very few lncRNA signatures have been developed for HCC prognosis prediction[26]. Thus, it is necessary to identify a more effective lncRNA signature for HCC prognosis. In the present study, we aimed to construct a lncRNA signature capable of predicting HCC prognosis with high efficiency.

In this work, we analyzed a cohort of 370 HCC patients from The Cancer Genome Atlas (TCGA) to identify a potential lncRNA signature for predicting the survival of HCC patients. We identified a five-lncRNA prognostic signature from the TCGA dataset and determined that its prognostic value was independent from clinical factors. The identification of prognostic lncRNAs suggests the potential roles of lncRNAs in HCC pathogenesis and progression.

MATERIALS AND METHODS

Data and patients
Level 3 RNA-seq data (HTSeq-counts) from 374 HCC tumor specimens and 50 peritumoral liver specimens and their corresponding clinicopathological information were downloaded from the TCGA project (https://cancergenome.nih.gov/) on June 2, 2017. Because TCGA data are a community resource project, additional ethical approval was not acquired, and the present study adhered to TCGA publication guidelines and data access policies. After excluding the data without complete survival information, a total of 370 HCC

patients with complete follow up data were enrolled in our study and then randomly divided into a training set (*n* = 184) and test set (*n* = 186) using SPSS software (version 24.0). The clinicopathological parameters of the HCC patients in each group are listed in Table 1.

LncRNA expression profile in the TCGA LIHC cohort
Only lncRNAs with a description in NCBI or Ensemble were selected for further study in this paper. We obtained the expression profiles of 6929 lncRNAs from the RNA-seq data of the TCGA LIHC cohort. Differentially expressed lncRNAs (DELs) between the HCC specimens and peritumor liver specimens were identified with the edgeR package, using an adjusted $P < 0.05$ and $\log_2$ |fold change| > 1. The expression level of each lncRNA was $\log_2$ transformed for the downstream analyses.

Identification of prognostic lncRNAs and construction of the risk formula for overall survival prediction
Univariate Cox proportional hazards regression was performed to obtain the DELs that were significantly associated with the overall survival (OS) of HCC patients in the training group. After acquiring survival-related lncRNAs ($P < 0.01$), we excluded those not expressed in at least 10% of the samples. The remaining OS-related lncRNAs were then adjusted sing the stepwise multivariate Cox regression model. Finally, those lncRNAs fitted in the multivariate Cox regression model and independently associated with OS were chosen. A
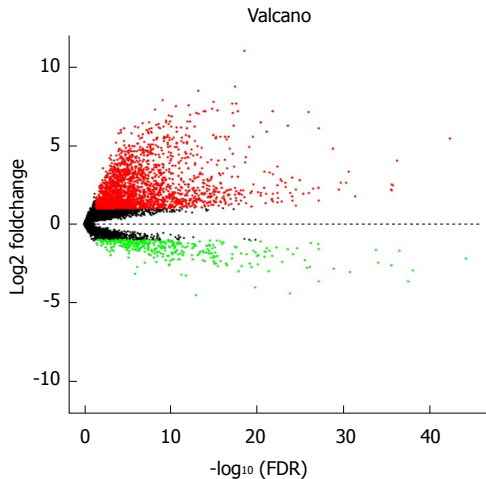
Valcano

Figure 1 Volcano plot of the differentially expressed long non-coding RNAs between hepatocellular carcinoma tumor specimens and peritumoral liver specimens. The X-axis represents the adjusted FDR, and the Y-axis represents the value of the $\log_2$ fold change. Aberrantly expressed long non-coding RNAs (lncRNAs) were calculated using the EdgeR package. Red dots represent upregulated lncRNAs in the hepatocellular carcinoma (HCC) tumor specimens, while green dots indicate downregulated lncRNAs compared with the peritumoral liver specimens. Black dots show the lncRNAs without significant differences between the HCC tumor and peritumoral liver specimens. Altogether, 2240 upregulated and 353 downregulated lncRNAs were found. This volcano plot was conducted using the ggplot2 package of R language.

prognostic risk formula was established based on a linear combination of the expression level of these lncRNAs multiplied by the regression coefficient derived from the multivariate Cox regression model as previously described[18-21]. The subjects in each dataset were classified into a high-risk group and low-risk group according to the median risk score of the risk formula derived from the training set.

Statistical analysis
Univariate Cox proportional hazards regression was performed to obtain survival-related DELs, and the stepwise multivariate Cox regression model was performed for further selection. Overall survival analyses in the high-risk and low-risk groups were performed using Kaplan-Meier survival curves and a log-rank test. Receiver operating curve analyses were performed to assess the specificity and sensitivity of the prognosis prediction. The above analyses were performed using R (version 3.3.1). To verify the independence of the prognostic value of the 5-lncRNA signature and clinicopathological parameters, univariate and multivariate Cox regression analyses were performed using SPSS software (version 24.0). In the comparison study, Kaplan-Meier survival analysis and receiver operating curve (ROC) analysis were also performed using SPSS (version 24.0).

Functional enrichment analyses
To identify co-expressed lncRNA-mRNA pairs, we performed Person correlation analyses with R (version

3.3.1) for each of the five lncRNAs with protein-coding genes based on the RNA-seq data of the TCGA LIHC cohort. The protein-coding genes with a correlation coefficient > 0.5 and a *P* < 0.01 were considered to be significantly correlated genes. For functional enrichment analysis, the correlated protein-coding genes were subjected to gene ontology (GO) and Kyoto Encyclopediaof Genes and Genomes (KEGG) pathway analyses using DAVID Bioinformatics Resources (version 6.8)[27,28]. Significant functional categories were identified and limited to GO terms in the "Biological Process" (GOTERM-BP-DIRECT) and KEGG pathway categories, using the human whole genome as the background. Significantly enriched GO terms with similar functions were visualized using the EnrichmentMap plugin in Cytoscape (version 3.5.1)[29].

RESULTS

Determining prognostic lncRNAs from the training set
Using the edgeR package, we identified a total of 2593 lncRNAs differentially expressed ($\log_2$|fold change| > 1 and adjusted *P* < 0.05) between 374 HCC tumor specimens and 50 peritumor liver specimens, including 2240 upregulated and 353 downregulated lncRNAs (Figure 1). A total of 370 HCC samples with complete survival information were subjected to further analyses. For the training set, univariate Cox proportional hazards regression analyses revealed 82 lncRNAs significantly correlated with OS (*P* < 0.01) among the 2593 differentially expressed lncRNAs. Among the 82 OS-related lncRNAs, we further excluded those expressed in less than 10% of the HCC specimens, and the remaining 30 lncRNAs were subjected to further selection.

Construction of a lncRNA-based prognostic signature and validation in the training group
Stepwise multivariable Cox proportional hazards regression analyses were performed to identify the optimal prognostic lncRNAs among the 30 candidate lncRNAs. Based on this model, a final 5 lncRNAs were found to be significantly and independently related to prognosis. We then constructed a prognostic signature based on the expression levels of these 5 lncRNAs and their coefficients derived from the multivariable Cox model. The formula is as follows: risk score = (-0.1900 × the expression level of AC015908.3) + (0.1764 × the expression level of FOXD2-AS1) + (0.3588 × the expression level of AC091057.3) + (0.5615 × the expression level of TMCC1-AS1) + (0.4877 × the expression level of DCST1-AS1). Detailed information for the 5 lncRNAs is listed in Table 2. The risk score for each patient in the training group was calculated using the formula. The training set was then divided into a high-risk group (*n* = 92) and a low-risk group (*n* = 92) according to the median risk score. Kaplan-Meier analysis revealed that the high-risk group had a significantly poorer prognosis than that of the low-

Table 2 The 5 long non-coding RNAs significantly associated with overall survival of hepatocellular carcinoma patients

Gene ID	Gene symbol	Coefficient	Hazard ratio	*P* value
ENSG00000264016	AC015908.3	-0.1900	0.7792	0.000305
ENSG00000237424	FOXD2-AS1	0.1764	1.2865	0.007317
ENSG00000269974	AC091057.3	0.3588	1.4682	0.000375
ENSG00000271270	TMCC1-AS1	0.5615	1.5417	0.000287
ENSG00000232093	DCST1-AS1	0.4877	1.3909	0.001632

risk group (P = 1.3e-09, log-rank test, Figure 2A). The median survival time for the high-risk group and the low-risk group was 2.096 and 6.811 years, respectively. Additionally, the 3- and 5-year survival rates of the high-risk group were 40% and 23.5%, whereas the corresponding survival rates were 90% and 71.8%, respectively, in the low-risk group. To evaluate the performance of the 5-lncRNA signature for predicting the prognosis of HCC patients, a time-dependent ROC analysis was conducted. The area under the ROC curve (AUC) for the 5-lncRNA signature was 0.857, which indicated good performance (Figure 2B). The risk scores of patients in the training group were also ranked, and survival status was plotted for each patient on a dot plot (Figure 2C). The mortality for patients in the high-risk group was much higher than that in the low-risk group. A heat map displays the expression profiles of these five lncRNAs in the samples from the training group; the expression profiles are ranked according to risk score (Figure 2D). Among the 5 lncRNAs, AC015908.3 showed a negative coefficient derived for the multivariate Cox regression model and seemed to be a protective factor, as its high expression predicts a low risk. The other 4 lncRNAs with positive coefficients, including FOXD2-AS1, AC091057.3, TMCC1-AS1 and DCST1-AS1, seemed to be risk factors and all were upregulated in the high-risk group compared to the low-risk group within the training set.

Validation of the prognostic value of the 5-lncRNA signature for the test set and the entire cohort

To further verify the prognostic value of the 5-lncRNA signature for HCC patients, risk scores for patients in the test group were calculated according to the constructed formula based on the expression of the 5 lncRNAs. The test group was also divided into high-risk (n = 97) and low-risk (n = 89) groups using the same cutoff as for the training group. Kaplan-Meier analysis revealed that the survival rate of the high-risk subgroup was much lower than that of the low-risk subgroup in the test set (median OS: 2.293 years *vs* 8.562 years; log-rank P = 1.64e-05) (Figure 3A). For the entire set, a similar result was obtained by Kaplan-Meier analysis. Among the entire cohort, the median survival of the high-risk group (n = 189) was 2.197 years, which was significantly lower than the median OS of 6.937 years for the low-risk group (n = 181) (P = 2.69e-13, Figure 4A). The AUC for the 5-lncRNA-based risk score of overall survival was 0.709 and 0.769 for

the test group (Figure 3B) and the entire group (Figure 4B), respectively, with both showing robust utility. In addition, ranked risk scores and survival status for each subject were plotted for the test group (Figure 3C) and the entire set (Figure 4C). Heatmaps display the expression profiles of the five lncRNAs for each subject in the test group (Figure 3D) and the entire cohort (Figure 4D), which were ranked according to risk score.

The prognostic value of the 5-lncRNA signature was independent of clinical characteristics

Univariate and multivariate Cox regression analyses were performed with the 5-lncRNA-based risk score and clinicopathological factors, including age, gender, weight, Child-Pugh grading, fibrosis extent, vascular tumor invasion, serum FAP levels, tumor grade and pathological stage as explanatory variables and overall survival as the dependent variable. The univariate Cox regression demonstrated that the 5-lncRNA signature-based risk score and pathologic stage were able to effectively predict the prognosis of HCC patients. In addition, in the training set and the entire set, patient age seemed to be related to survival, although this did not reach significance (Table 3). In contrast, none of the other clinicopathological parameters were associated with prognosis in either set. Multivariate Cox regression analysis revealed that after adjusting for other factors, age (only for the entire set), pathologic stage and the 5-lncRNAsignature were the only factors significantly associated with overall survival (Table 2). Patients from the entire cohort were then stratified by age (Figure 5A) and pathological stage (Figure 5B). Each subgroup was then divided into a high-risk and low-risk group based on the 5-lncRNA risk score median derived from the training group. Kaplan-Meier analysis revealed that for all of the subgroups, the high-risk group had significantly poorer survival than the low-risk group. All of these results strongly suggest that the prognostic value of the 5-lncRNA-based risk score is independent of clinicopathological factors.

Comparison of the 5-lncRNA signature with existing prognostic signatures for HCC

Two HCC-related prognostic signatures have recently been developed and reported, including a 3-gene signature by Binghua Li and a 4-lncRNA signature by Zhonghao Wang that were both derived from the TCGA dataset[25,26]. To compare the prognostic value of the 5-lncRNA signature developed in our present study
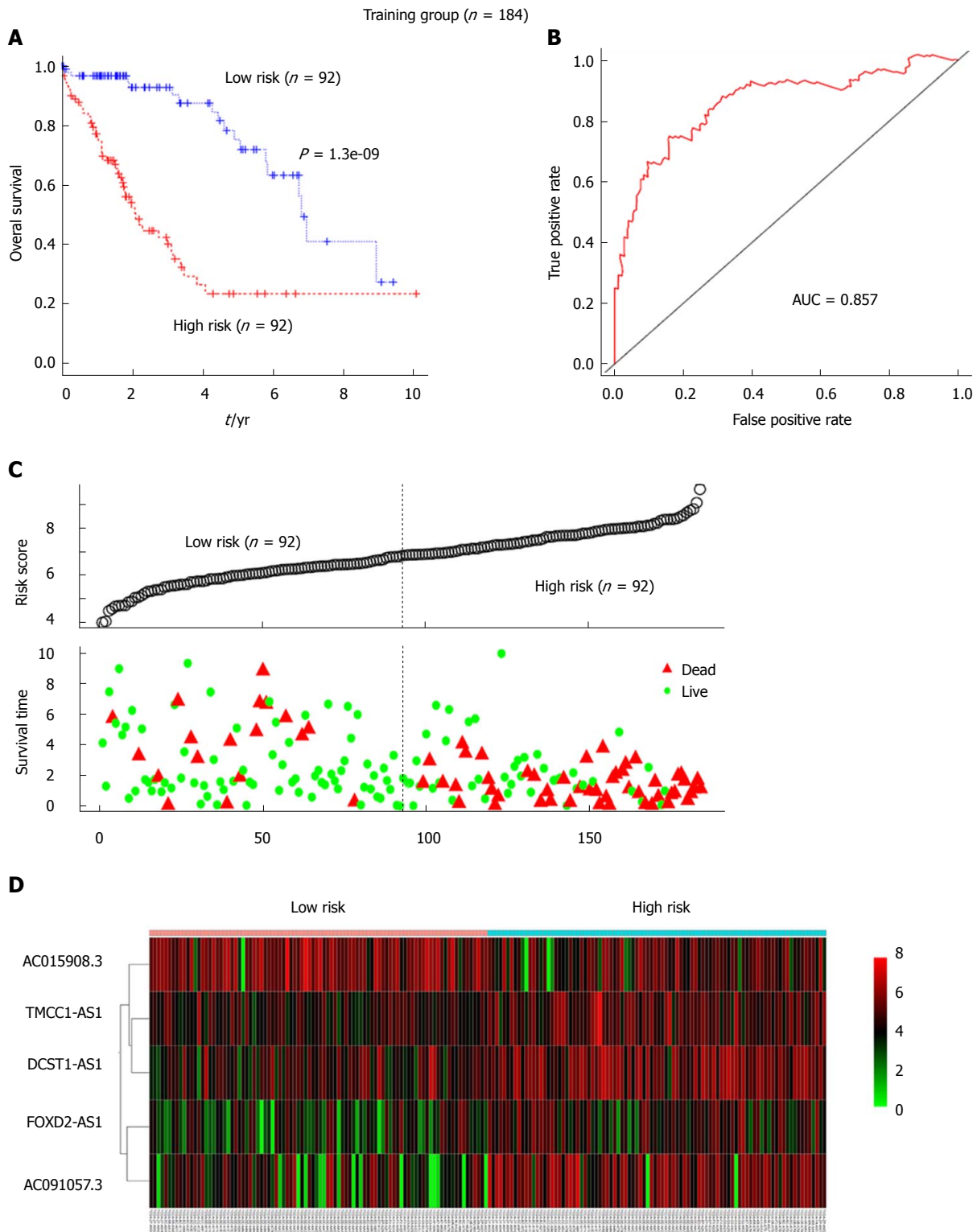
Figure 2 The 5-long non-coding RNA signature-based risk score predicted the overall survival of patients with hepatocellular carcinoma in the training set (*n* = 184). A: Kaplan-Meier analysis of patients' overall survival in the high-risk (*n* = 92) and low-risk (*n* = 92) subgroups of the training set; B: The receiver operating characteristic (ROC) analysis of the risk score for prediction the overall survival of the training set. The area under the curve was calculated for ROC curves; C: The 5-lncRNA-based risk score distribution, patient survival status; D: Heatmap of the 5-lncRNA expression profiles in the high-risk and low-risk subgroups for the training set.

(hereafter referred to as 5LncSig) with the existing 3-gene signature by Binghua Li (hereafter referred to as 3GeneSig) and the 4-lncRNA signature by Zhonghao Wang (hereafter referred to as ZhongSig), we calculated the risk scores of each patient in the entire cohort based on formulae derived from each of these signatures.

The 3GeneSig and ZhongSig both successfully and significantly predicted prognosis in the entire TCGA LIHC cohort (Figure 6A and B). Furthermore, comparison of the Kaplan-Meier curves revealed that patients in the high-risk group predicted by 5LncSig showed a dramatically poorer prognosis than those in the low-

Table 3 Univariate and multivariate Cox regression analysis of overall survival

Variables	Univariate analysis			Multivariate analysis		
	HR	95%CI of HR	*P* value	HR	95%CI of HR	*P* value
Training set (*n* = 184)						
Risk score	2.718	2.093-3.530	< 0.0001	2.830	2.091-3.831	< 0.0001
Age	1.018	0.998-1.038	0.073			
Sex (Male/Female)	1.022	0.619-1.690	0.931			
Weight	1.006	0.993-1.108	0.397			
Child-Pugh grade	0.845	0.253-2.827	0.195			
Fibrosis ishak score	0.685	0.756-1.283	0.910			
Vascular invasion (yes/no)	0.834	0.480-1.449	0.519			
FAP	0.805	0.932-1.056	0.805			
Tumor grade						
(G1 + G2/G3 + G4)	1.133	0.680-1.887	0.632			
Pathologic stage				1.900	1.098-3.288	0.022
I / II	0.604	0.303-1.203	0.152			
I / III + IV	0.298	0.163-0.543	< 0.0001			
Test set (*n* = 186)						
Risk score	1.603	1.270-2.024	< 0.0001	1.568	1.196-2.055	**0.001**
Age	1.006	0.987-1.026	0.522			
Sex (Male/Female)	1.420	0.856-2.356	0.174			
Weight	1.000	0.984-1.105	0.960			
Child-Pugh grade	0.475	0.195-1.157	0.101			
Fibrosis ishak score	1.640	0.706-3.813	0.250			
Vascular invasion (yes/no)	1.069	0.613-1.866	0.614			
FAP	1.035	0.981-1.093	0.210			
Tumor grade						
(G1 + G2/G3 + G4)	1.092	0.655-1.818	0.736			
Pathologic stage				2.103	1.193-3.707	0.010
I / II	0.865	0.426-1.757	0.688			
I / III + IV	0.445	0.250-0.793	0.006			
Entire set (*n* = 370)						
Risk score	1.957	1.646-2.327	< 0.0001	2.011	1.638-2.469	< 0.0001
Age	1.013	0.999-1.027	0.068	1.016	1.000-1.032	0.048
Sex (Male/Female)	1.166	0.817-1.664	0.396			
Weight	0.998	0.988-1.007	0.615			
Child-Pugh grade (A/B + C)	0.620	0.306-1.256	0.184			
Fibrosis ishak score	1.232	0.742-2.045	0.365			
Vascular invasion (yes/no)	0.962	0.650-1.424	0.846			
Serum AFP level	1.023	0.980-1.068	0.306			
Tumor grade						
(G1 + G2/G3 + G4)	1.119	0.780-1.604	0.542			
Pathologic stage				2.017	1.359-2.993	0.027
I / II	0.648	0.398-1.056	0.082			
I / III + IV	0.351	0.236-0.524	< 0.0001			

risk groups predicted by the 3GeneSig and ZhongSig (Figure 6A and B), and patients in the low-risk group predicted by 5LncSig had a much better prognosis than those in the high-risk group predicted by the other two signatures (Figure 6A and B). To compare the sensitivity and specificity of the 5LncSig for prognosis prediction with the other two existing signatures, we performed time-dependent ROC analysis. The AUC of overall survival for the 3GeneSig and the ZhongSig was 0.701 and 0.721, respectively (Figure 6C), both lower than that of the 5LncSig (0.769). Thus, the prognostic power of 5LncSig, developed in the present study, was superior to that of the previously developed 3-gene and 4-lncRNA signatures.

Functional characteristics of the five prognostic lncRNAs

To explore the functional implications of these 5 lncRNAs,

we performed Pearson correlation analyses between the 5 lncRNAs and protein-coding genes based on their expression levels in the TCGA LIHC cohort. The protein-coding genes that correlated with at least 1 of the 5 lncRNAs (Pearson coefficient > 0.5, P < 0.01) were considered to be correlated genes. We chose the 200 correlated genes with the highest Pearson coefficients for further analysis. Functional enrichment analysis revealed that these genes were primarily enriched in 32 GO terms (Benjamin P value < 0.1, Figure 7A) and 23 KEGG pathways (P < 0.001, Figure 7B). Further analysis revealed that these enriched GO functional terms are mostly involved in metabolic processes, fibrinolysis and complement activation (Figure 7A).

DISCUSSION

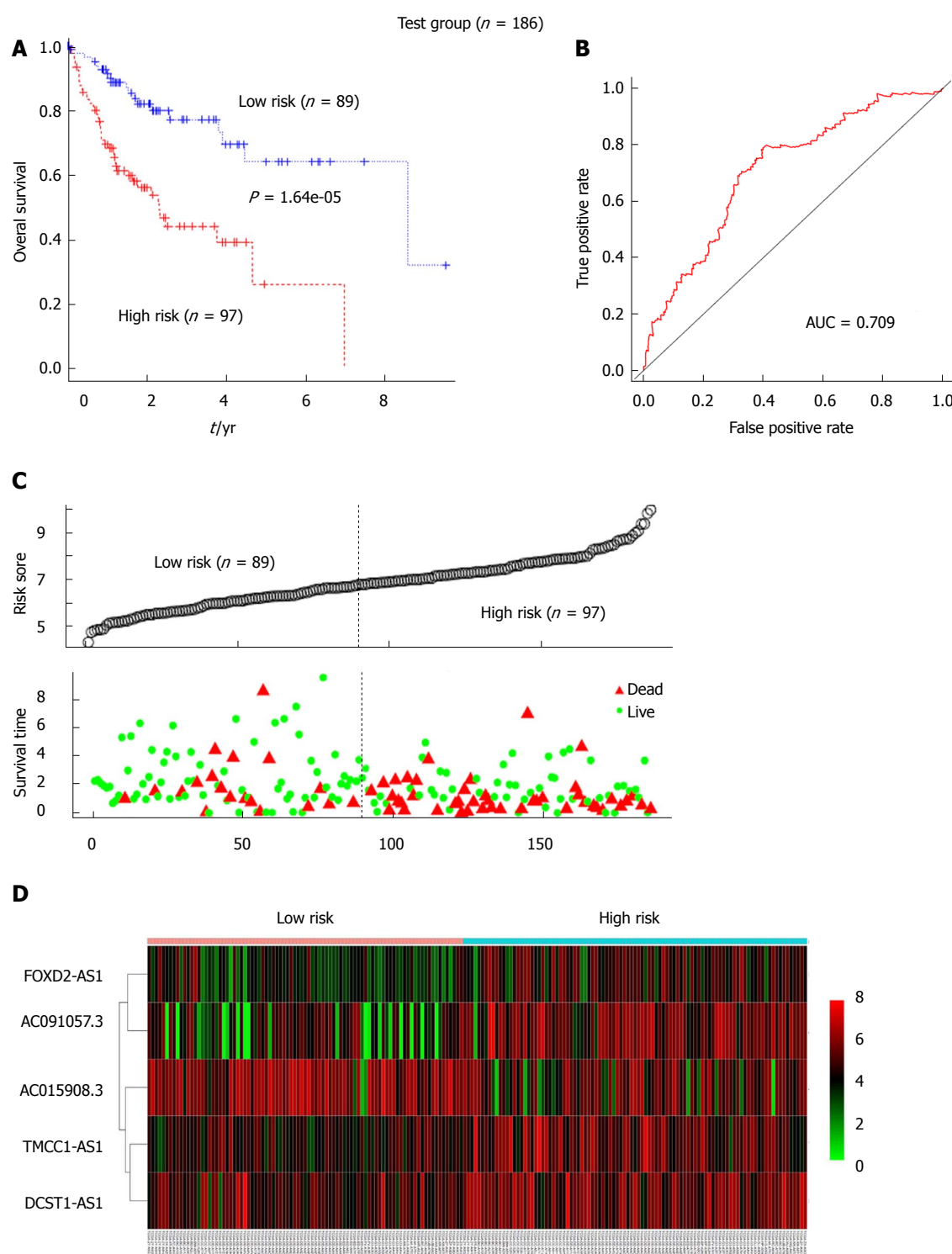HCC is a heterogeneous disease with differential

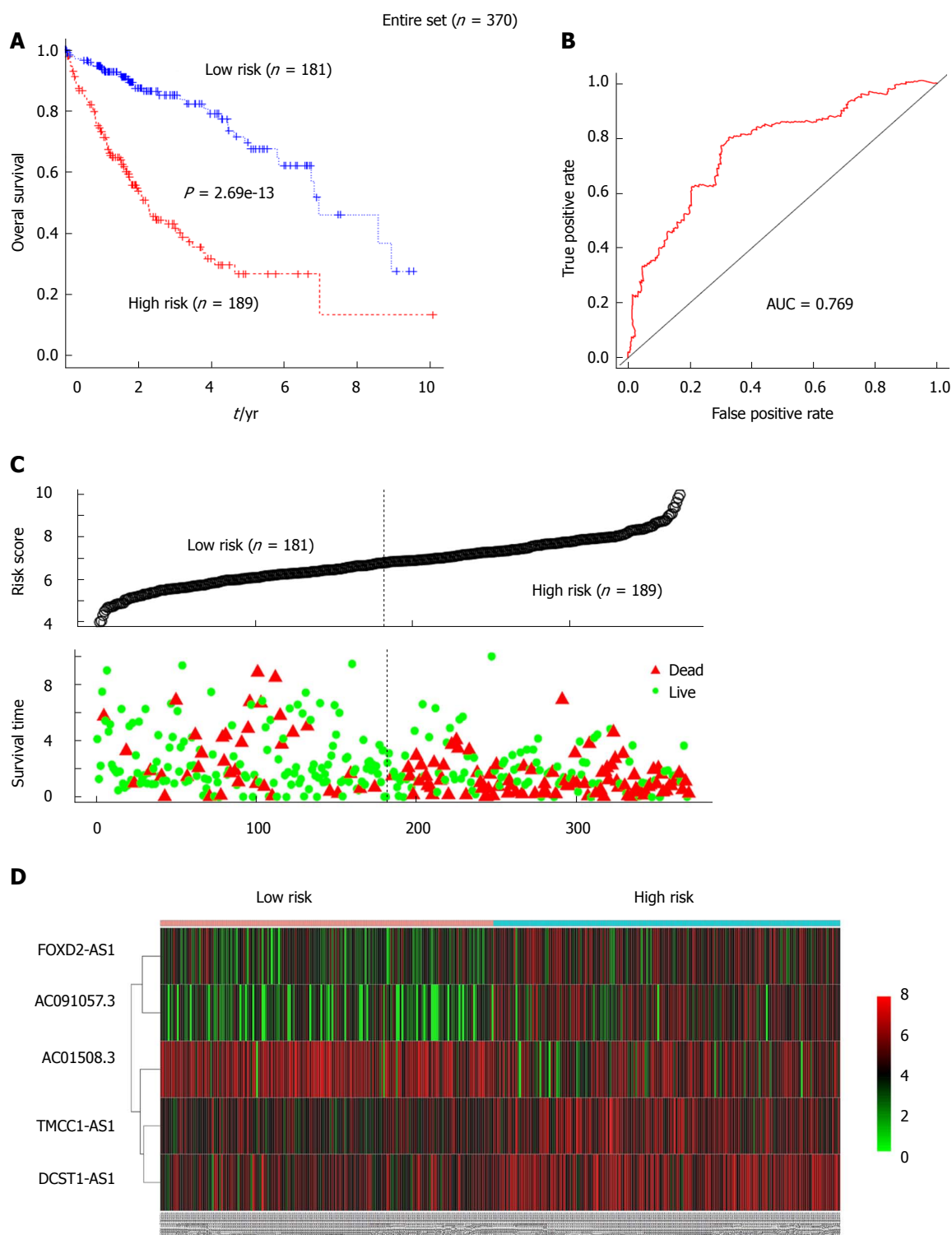**Figure 3 Validation of the prognostic value of the 5-long non-coding RNA signature for the hepatocellular carcinoma patients in the test group.** A: Kaplan-Meier analysis indicated that patients in the high-risk (*n* = 97) subgroup exhibited significantly poorer survival than the low-risk subgroup (*n* = 89) in the test group; B: The receiver operating characteristic analysis of the risk score for predicting the overall survival of the test set; C: The 5-long non-coding RNA (lncRNA)-based risk score distribution, patient survival status; D: Heatmap of the 5-lncRNA expression profiles in the high-risk and low-risk subgroups of the test set.

prognoses and a high mortality. Until now, no biomarkers have been shown to effectively predict the survival of HCC patients, and thus, finding effective biomarkers for HCC is crucial.

Previous investigations of gene regulation and disease pathogenesis have mainly focused on protein-coding genes, which account for only a very small proportion

(2%) of transcribed genes in eukaryotic species[13]. Recent developments in genome and transcriptome sequencing technologies have profoundly expanded our knowledge of non-coding RNAs, which are much more abundant than canonical protein-coding mRNAs[30,31]. Multiple studies indicate that lncRNAs act not only as intermediaries between DNA and protein but also

Figure 4 The prognostic value of the 5-long non-coding RNA signature for the hepatocellular carcinoma patients in the entire set (*n* = 370). A: Kaplan-Meier analysis of patients' overall survival in the high-risk (*n* = 189) and low-risk subgroups (*n* = 181) for the entire set; B: The receiver operating characteristic analysis of the risk score for predicting the overall survival of the test set; C: The 5-long non-coding RNA (5-lncRNA)-based risk score distribution, patient survival status; D: Heatmap of the 5-lncRNA expression profiles in the high-risk and low-risk subgroups for the entire set.

as important regulators of diverse cellular functions. lncRNAs have been shown to regulate the expression and function of protein-coding genes at the chromatin, transcriptional and post-transcriptional levels[31]. Many studies have revealed the contribution of lncRNAs in cancer development, indicating their potential as novel biomarkers for cancer diagnosis and prognosis[32-35].

LncRNA signatures for prognostic prediction prognoses have been developed for many cancers including renal cancer, glioblastoma, and colorectal cancer, among others[18-21]. Regarding HCC, the existing gene signatures for survival prediction have focused mostly on mRNAs and microRNAs. Several potential lncRNA biomarkers associated with the progression and

A

B

Figure 5 Stratification analyses of the prognostic value of the 5-long non-coding RNA signature-based risk score for all 370 hepatocellular carcinoma patients in the entire set. A: Kaplan-Meier analysis of the overall survival of hepatocellular carcinoma patients < 60 or ≥ 60 years old; B: Kaplan-Meier analysis of the overall survival of patients with different pathological stages.

prognosis of HCC have been identified, such as TSLNC8, HOXD-AS1 and CACS2[36-38]. These lncRNAs are thought to impact HCC progression through their regulation of tumor cell proliferation, EMT, apoptosis and migration. Although many of these lncRNAs are closely associated

with the prognosis and survival of HCC patients, their prognostic value has been tested only in small-scale studies; they have not yet been validated in a large clinical cohort. Until now, relatively few comprehensive lncRNA signatures for the prediction of HCC survival

Figure 6 Comparison of the sensitivity and specificity for hepatocellular carcinoma survival prediction by the 5-long non-coding RNA signature and two existing signatures in the entire set. A: Kaplan-Meier analysis of patients' overall survival in the high-risk (n = 185) and low-risk (n = 185) subgroups based on the 3GeneSig in the entire set (left panel) and comparison of the survival difference based on the 5 long non-coding RNA (lncRNA) signature and the 3GeneSig by Kaplan-Meier analysis (right panel); B: Kaplan-Meier analysis of patients' overall survival in the high-risk (n = 186) and low-risk (n = 184) subgroups based on the ZhongSig in the entire set (left panel) and comparison of the survival difference based on the 5 lncRNA signature and the ZhongSig (right panel); C: The ROC analysis of overall survival for the 5-lncRNA signature, ZhongSig and 3GeneSig.

have been constructed[26]. TCGA is an open-access database including samples from hundreds of patients with various malignancies. In the present study, we downloaded the RNA sequencing data of the TCGA LIHC

cohort and acquired lncRNA expression profiles for HCC patients in the dataset. Using univariate and stepwise multivariate Cox regression analyses, we developed a prognostic formula for HCC based on the expression

Figure 7 **Functional enrichment analysis of the 5-long non-coding RNA based on their correlated protein-coding genes.** A: Functional enrichment map of significantly enriched gene ontology terms; B: KEGG pathways significantly associated with the correlated protein-coding genes.

of 5 lncRNAs including AC015908.3, AC091057.3, TMCC1-AS1, DCST1-AS1 and FOXD2-AS1. In the

training set, HCC patients with high-risk scores based on the 5-lncRNA signature had a significantly reduced

survival time compared to those with low-risk scores. The prognostic value of the 5-lncRNA signature for HCC patients was further validated in the test group and the entire group, with robust and reproducible predictive indices. The results of these analyses suggest that the prognostic value of the 5-lncRNA-based risk model is robust and reliable for predicting survival in HCC patients.

In the present study, when adjusted using multivariate Cox regression analyses, age (only for the entire set), pathological stage and the 5-lncRNA signature were shown to independently predict the survival of HCC patients. The results of stratification analyses demonstrated that the prognostic value of the 5-lncRNA signature remained significant and robust in HCC subgroups stratified by age and pathological stage. In an attempt to further validate its prognostic value in other HCC cohorts, we downloaded data from several GEO datasets. Unfortunately, most of the 5 lncRNA probes could not be found. There are many existing prognostic signatures for HCC, and we therefore compared our 5-lncRNA signature with two recently developed signatures: a 3-gene signature and a 4-lncRNA signature. The results indicate that the predictive performance of the 5-lncRNA signature was superior to that of the other two signatures for HCC overall survival.

To the best of our knowledge, the functions of these 5 lncRNAs have not been reported. Functional enrichment analysis revealed that the protein-coding genes that were significantly correlated with these 5lncRNAs are enriched for metabolic processes, fibrinolysis and complement activation. KEGG pathway analysis revealed that these genes are enriched in pathways related to metabolism. These results suggest that the 5 lncRNAs may participate in the initiation and progression of HCC through these pathways. However, further studies are needed to investigate and validate the functions of these 5 lncRNAs.

In conclusion, our present study developed a 5-lncRNA signature for predicting the prognosis of HCC patients. The signature was reproducible and robust in a second independent large-scale HCC cohort, supporting its value and effectiveness. In addition, the prognostic value of the 5-lncRNA signature was independent of clinicopathological variables. Our study indicates that the 5-lncRNA signature could improve survival prediction and could be used as a prognostic biomarker for HCC patients.

ARTICLE HIGHLIGHTS

Research background
Hepatocellular carcinoma (HCC) is the sixth most commonly diagnosed cancer in the world. Although treatment for HCC, including surgical resection, has improved over the past decades, its overall survival rate remains devastatingly high due to its high rate of recurrence. Because HCC is a heterogeneous disease with substantially variable clinical outcomes, the search for effective biomarkers to predict recurrence and prognosis is crucial.

Research motivation
Recent studies have demonstrated the importance of long non-coding RNAs (lncRNAs) in physiological and pathological cellular processes. Increasing evidence suggests that lncRNA dysregulation is associated with various human diseases, particularly the initiation and progression of various human cancers. For patients with HCC, most of the existing prognostic signatures have focused on mRNAs or microRNAs, and only a few lncRNA signatures have been developed. In the present study, we aimed to construct a lncRNA signature for the prediction of HCC prognosis with high efficiency.

Research objectives
To construct a lncRNA signature for the prediction of HCC prognosis with high efficiency.

Research methods
Differentially expressed lncRNAs (DELs) between HCC specimens and peritumor liver specimens were acquired from the The Cancer Genome Atlas (TCGA) LIHC dataset using the edgeR package. Univariate Cox proportional hazards regression was performed to identify the DELs that were significantly associated with overall survival for the training set. The stepwise multivariate Cox regression model was applied. Those lncRNAs fitted in the multivariate Cox regression model and independently associated with overall survival were chosen to build a prognostic risk formula. The prognostic value of this formula was validated in the test group and the full cohort and further compared with two previously developed prognostic signatures for HCC.

Research results
We identified a five-lncRNA prognostic signature from the TCGA dataset and determined that its prognostic value was independent from clinicopathological factors. The signature was reproducible and robust in another independent large-scale HCC cohort, supporting its utility and effectiveness.

Research conclusions
This study constructed a 5-lncRNA signature that improves survival prediction, and can be used as a prognostic biomarker for HCC patients.

REFERENCES

1   **Ferlay J**, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015; **136**: E359-E386 [PMID: 25220842 DOI: 10.1002/ijc.29210]

2   **Torre LA**, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**: 87-108 [PMID: 25651787 DOI: 10.3322/caac.21262]

3   **Venook AP**, Papandreou C, Furuse J, de Guevara LL. The incidence and epidemiology of hepatocellular carcinoma: a global and regional perspective. *Oncologist* 2010; **15** Suppl 4: 5-13 [PMID: 21115576 DOI: 10.1634/theoncologist.2010-S4-05]

4   **Allemani C**, Weir HK, Carreira H, Harewood R, Spika D, Wang XS, Bannon F, Ahn JV, Johnson CJ, Bonaventure A, Marcos-Gragera R, Stiller C, Azevedo e Silva G, Chen WQ, Ogunbiyi OJ, Rachet B, Soeberg MJ, You H, Matsuda T, Bielska-Lasota M, Storm H, Tucker TC, Coleman MP; CONCORD Working Group. Global surveillance of cancer survival 1995-2009: analysis of individual data for 25,676,887 patients from 279 population-based registries in 67 countries (CONCORD-2). *Lancet* 2015; **385**: 977-1010 [PMID: 25467588 DOI: 10.1016/S0140-6736(14)62038-9]

5   **Mittal S**, El-Serag HB. Epidemiology of hepatocellular carcinoma: consider the population. *J Clin Gastroenterol* 2013; **47** Suppl: S2-S6 [PMID: 23632345 DOI: 10.1097/MCG.0b013e3182872f29]

6   **El-Serag HB**, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007; **132**: 2557-2576 [PMID: 17570226 DOI: 10.1053/j.gastro.2007.04.061]

7   **Joliat GR**, Allemann P, Labgaa I, Demartines N, Halkic N.

Treatment and outcomes of recurrent hepatocellular carcinomas. *Langenbecks Arch Surg* 2017; **402**: 737-744 [PMID: 28497194 DOI: 10.1007/s00423-017-1582-9]

8   **Bruix J**, Gores GJ, Mazzaferro V. Hepatocellular carcinoma: clinical frontiers and perspectives. *Gut* 2014; **63**: 844-855 [PMID: 24531850 DOI: 10.1136/gutjnl-2013-306627]

9   **Zheng J**, Kuk D, Gönen M, Balachandran VP, Kingham TP, Allen PJ, D'Angelica MI, Jarnagin WR, DeMatteo RP. Actual 10-Year Survivors After Resection of Hepatocellular Carcinoma. *Ann Surg Oncol* 2017; **24**: 1358-1366 [PMID: 27921192 DOI: 10.1245/s10434-016-5713-2]

10   **Kudo M**. Surveillance, diagnosis, treatment, and outcome of liver cancer in Japan. *Liver Cancer* 2015; **4**: 39-50 [PMID: 26020028 DOI: 10.1159/000367727]

11   **Bruix J**, Sherman M; American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53**: 1020-1022 [PMID: 21374666 DOI: 10.1002/hep.24199]

12   **Schmitt AM**, Chang HY. Long Noncoding RNAs: At the Intersection of Cancer and Chromatin Biology. *Cold Spring Harb Perspect Med* 2017; **7**: [PMID: 28193769 DOI: 10.1101/cshperspect.a026492]

13   **Hangauer MJ**, Vaughn IW, McManus MT. Pervasive transcription of the human genome produces thousands of previously unidentified long intergenic noncoding RNAs. *PLoS Genet* 2013; **9**: e1003569 [PMID: 23818866 DOI: 10.1371/journal.pgen.1003569]

14   **Mercer TR**, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. *Nat Rev Genet* 2009; **10**: 155-159 [PMID: 19188922 DOI: 10.1038/nrg2521]

15   **Moran VA**, Perera RJ, Khalil AM. Emerging functional and mechanistic paradigms of mammalian long non-coding RNAs. *Nucleic Acids Res* 2012; **40**: 6391-6400 [PMID: 22492512 DOI: 10.1093/nar/gks296]

16   **Hauptman N**, Glavač D. Long non-coding RNA in cancer. *Int J Mol Sci* 2013; **14**: 4655-4669 [PMID: 23443164 DOI: 10.3390/ijms14034655]

17   **Gibb EA**, Brown CJ, Lam WL. The functional role of long non-coding RNA in human carcinomas. *Mol Cancer* 2011; **10**: 38 [PMID: 21489289 DOI: 10.1186/1476-4598-10-38]

18   **Shi D**, Qu Q, Chang Q, Wang Y, Gui Y, Dong D. A five-long non-coding RNA signature to improve prognosis prediction of clear cell renal cell carcinoma. *Oncotarget* 2017; **8**: 58699-58708 [PMID: 28938589 DOI: 10.18632/oncotarget.17506]

19   **Zhou M**, Zhang Z, Zhao H, Bao S, Cheng L, Sun J. An Immune-Related Six-lncRNA Signature to Improve Prognosis Prediction of Glioblastoma Multiforme. *Mol Neurobiol* 2018; **55**: 3684-3697 [PMID: 28527107 DOI: 10.1007/s12035-017-0572-9]

20   **Zeng JH**, Liang L, He RQ, Tang RX, Cai XY, Chen JQ, Luo DZ, Chen G. Comprehensive investigation of a novel differentially expressed lncRNA expression profile signature to assess the survival of patients with colorectal adenocarcinoma. *Oncotarget* 2017; **8**: 16811-16828 [PMID: 28187432 DOI: 10.18632/oncotarget.15161]

21   **Sun J**, Cheng L, Shi H, Zhang Z, Zhao H, Wang Z, Zhou M. A potential panel of six-long non-coding RNA signature to improve survival prediction of diffuse large-B-cell lymphoma. *Sci Rep* 2016; **6**: 27842 [PMID: 27292966 DOI: 10.1038/srep27842]

22   **Lin Z**, Xu SH, Wang HQ, Cai YJ, Ying L, Song M, Wang YQ, Du SJ, Shi KQ, Zhou MT. Prognostic value of DNA repair based stratification of hepatocellular carcinoma. *Sci Rep* 2016; **6**: 25999 [PMID: 27174663 DOI: 10.1038/srep25999]

23   **Lu M**, Kong X, Wang H, Huang G, Ye C, He Z. A novel microRNAs expression signature for hepatocellular carcinoma diagnosis and prognosis. *Oncotarget* 2017; **8**: 8775-8784 [PMID:

24   **Borel F**, Konstantinova P, Jansen PL. Diagnostic and therapeutic potential of miRNA signatures in patients with hepatocellular carcinoma. *J Hepatol* 2012; **56**: 1371-1383 [PMID: 22314424 DOI: 10.1016/j.jhep.2011.11.026]

25   **Li B**, Feng W, Luo O, Xu T, Cao Y, Wu H, Yu D, Ding Y. Development and Validation of a Three-gene Prognostic Signature for Patients with Hepatocellular Carcinoma. *Sci Rep* 2017; **7**: 5517 [PMID: 28717245 DOI: 10.1038/s41598-017-04811-5]

26   **Wang Z**, Wu Q, Feng S, Zhao Y, Tao C. Identification of four prognostic LncRNAs for survival prediction of patients with hepatocellular carcinoma. *PeerJ* 2017; **5**: e3575 [PMID: 28729955 DOI: 10.7717/peerj.3575]

27   **Huang da W**, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* 2009; **37**: 1-13 [PMID: 19033363 DOI: 10.1093/nar/gkn923]

28   **Huang da W**, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009; **4**: 44-57 [PMID: 19131956 DOI: 10.1038/nprot.2008.211]

29   **Merico D**, Isserlin R, Stueker O, Emili A, Bader GD. Enrichment map: a network-based method for gene-set enrichment visualization and interpretation. *PLoS One* 2010; **5**: e13984 [PMID: 21085593 DOI: 10.1371/journal.pone.0013984]

30   **Chen JA**, Conn S. Canonical mRNA is the exception, rather than the rule. *Genome Biol* 2017; **18**: 133 [PMID: 28687088 DOI: 10.1186/s13059-017-1268-1]

31   **Shi X**, Sun M, Liu H, Yao Y, Song Y. Long non-coding RNAs: a new frontier in the study of human diseases. *Cancer Lett* 2013; **339**: 159-166 [PMID: 23791884 DOI: 10.1016/j.canlet.2013.06.013]

32   **Bi M**, Yu H, Huang B, Tang C. Long non-coding RNA PCAT-1 over-expression promotes proliferation and metastasis in gastric cancer cells through regulating CDKN1A. *Gene* 2017; **626**: 337-343 [PMID: 28571676 DOI: 10.1016/j.gene.2017.05.049]

33   **Hua F**, Liu S, Zhu L, Ma N, Jiang S, Yang J. Highly expressed long non-coding RNA NNT-AS1 promotes cell proliferation and invasion through Wnt/β-catenin signaling pathway in cervical cancer. *Biomed Pharmacother* 2017; **92**: 1128-1134 [PMID: 28628975 DOI: 10.1016/j.biopha.2017.03.057]

34   **Xu S**, Kong D, Chen Q, Ping Y, Pang D. Oncogenic long noncoding RNA landscape in breast cancer. *Mol Cancer* 2017; **16**: 129 [PMID: 28738804 DOI: 10.1186/s12943-017-0696-6]

35   **Spurlock CF 3rd**, Shaginurova G, Tossberg JT, Hester JD, Chapman N, Guo Y, Crooke PS 3rd, Aune TM. Profiles of Long Noncoding RNAs in Human Naive and Memory T Cells. *J Immunol* 2017; **199**: 547-558 [PMID: 28600289 DOI: 10.4049/jimmunol.1700232]

36   **Zhang J**, Li Z, Liu L, Wang Q, Li S, Chen D, Hu Z, Yu T, Ding J, Li J, Yao M, Huang S, Zhao Y, He X. Long noncoding RNA TSLNC8 is a tumor suppressor that inactivates the interleukin-6/STAT3 signaling pathway. *Hepatology* 2018; **67**: 171-187 [PMID: 28746790 DOI: 10.1002/hep.29405]

37   **Lu S**, Zhou J, Sun Y, Li N, Miao M, Jiao B, Chen H. The noncoding RNA HOXD-AS1 is a critical regulator of the metastasis and apoptosis phenotype in human hepatocellular carcinoma. *Mol Cancer* 2017; **16**: 125 [PMID: 28724429 DOI: 10.1186/s12943-017-0676-x]

38   **Wang Y**, Liu Z, Yao B, Li Q, Wang L, Wang C, Dou C, Xu M, Liu Q, Tu K. Long non-coding RNA CASC2 suppresses epithelial-mesenchymal transition of hepatocellular carcinoma cells through CASC2/miR-367/FBXW7 axis. *Mol Cancer* 2017; **16**: 123 [PMID: 28716020 DOI: 10.1186/s12943-017-0702-z]

ORIGINAL ARTICLE

Retrospective Study

Application of modified primary closure of the pelvic floor in laparoscopic extralevator abdominal perineal excision for low rectal cancer

Yan-Lei Wang, Xiang Zhang, Jia-Jia Mao, Wen-Qiang Zhang, Hao Dong, Fan-Pei Zhang, Shuo-Hui Dong, Wen-Jie Zhang, Yong Dai

Yan-Lei Wang, Xiang Zhang, Jia-Jia Mao, Wen-Qiang Zhang, Hao Dong, Fan-Pei Zhang, Shuo-Hui Dong, Wen-Jie Zhang, Yong Dai, Department of Colorectal and Anal Surgery, Qilu Hospital of Shandong University, Jinan 250012, Shandong Province, China

ORCID number: Yan-Lei Wang (0000-0003-1420-9529); Xiang Zhang (0000-0001-7417-6082); Jia-Jia Mao (0000-0002-2210-8456); Wen-Qiang Zhang (0000-0003-1162-2350); Hao Dong (0000-0002-7185-9002); Fan-Pei Zhang (0000-0001-9276-8436); Shuo-Hui Dong (0000-0002-2912-8046); Wen-Jie Zhang (0000-0003-1976-1523); Yong Dai (0000-0001-6163-8022).

Institutional review board statement: This study was approved by the Ethics Committee of Scientific Research of Shandong University Qilu Hospital.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest related to this study.

Data sharing statement: No additional data are available.

Correspondence to: Yong Dai, MD, PhD, Attending Doctor, Chief Doctor, Surgeon, Department of Colorectal and Anal Surgery, Qilu Hospital of Shandong University, No. 107 West Wenhua Road, Jinan 250012, Shandong Province, China. yongdai@hotmail.com
Telephone: +86-531-82166391

Abstract

AIM

To introduce a novel, modified primary closure technique of laparoscopic extralevator abdominal perineal excision (LELAPE) for low rectal cancer.

METHODS

We retrospectively analyzed data from 76 patients with rectal cancer who underwent LELAPE from March 2013 to May 2016. Patients were classified into the modified primary closure group (32 patients) and the biological mesh closure group (44 patients). The total operating time, reconstruction time, postoperative stay duration, total cost, postoperative complications and tumor recur-

rence were compared.

RESULTS

All surgery was successfully performed. The pelvic reconstruction time was 14.6 ± 3.7 min for the modified primary closure group, which was significantly longer than that of the biological mesh closure group (7.2 ± 1.9 min, $P < 0.001$). The total operating time was not different between the two groups (236 ± 20 min *vs* 248 ± 43 min, $P = 0.143$). The postoperative hospital stay duration was 8.1 ± 1.9 d, and the total cost was 9297 ± 1260 USD for the modified primary closure group. Notably, both of these categories were significantly lower in this group than those of the biological mesh closure group ($P = 0.001$ and $P = 0.003$, respectively). There were no differences observed between groups when comparing other perioperative data, long-term complications or oncological outcomes.

CONCLUSION

The modified primary closure method for reconstruction of the pelvic floor in LELAPE for low rectal cancer is technically feasible, safe and cost-effective.

Key words: Extralevator abdominoperineal excision; Rectal cancer; Pelvic floor; Laparoscopy

Core tip: The modified primary closure approach requires laparoscopic closure of the pelvic peritoneum and layered closure of the perineal defect. By using this modified approach, the length of hospital stay and the total cost were decreased significantly, while other clinical outcomes did not differ, except for a relatively longer time for pelvic reconstruction (14.6 ± 3.7 min *vs* 7.2 ± 1.9 min). We conclude that the modified primary closure method for reconstruction of the pelvic floor in laparoscopic extralevator abdominal perineal excision for low rectal cancer is technically feasible, safe and cost-effective.

INTRODUCTION

To improve the oncological outcome of patients with low rectal cancer, extralevator abdominal perineal excision (ELAPE) has been introduced to reduce the rate of positive circumferential margin and intraoperative perforation[1-4]. Assisted by laparoscopy, ELAPE can minimize physical invasion while ensuring oncological

benefits[5,6]. However, the extended resection of ELAPE may increase the risk of severe perineal wound complications, including perineal hernia, with a reported incidence of 20%-26%[7]. Thus, how to reconstruct the pelvic floor and close the perineum after massive resection has become a major concern and challenge in laparoscopic ELAPE (LELAPE). The established reconstruction methods include: primary perineal closure, omentoplasty, biological or synthetic mesh placement, myocutaneous flaps, and negative wound pressure therapy[8-12]. These methods all have their own advantages as well as restrictions, and no consensus has been reached so far. In traditional abdominoperineal resection (APR), the pelvic peritoneum is usually closed prior to reconstruction of the pelvic floor, in order to separate the small intestine from the presacral operating field, which is technically challenging in LELAPE. We have recently modified the primary closure technique by adding the laparoscopic pelvic peritoneum suture procedure, and applied it to LELAPE. In the present study, we compare this method with biological mesh closure in the reconstruction of the pelvic floor after LELAPE, and evaluate its feasibility, safety and cost-effectiveness.

MATERIALS AND METHODS

Study design

We retrospectively analyzed the data from 76 patients with rectal cancer undergoing LELAPE from March 2013 to May 2016. Patients were classified into the modified primary closure group (32 patients) and the biological mesh closure group (44 patients). Total operating time, reconstruction time, postoperative stay duration, total cost, postoperative complications and tumor recurrence were compared. The protocol was approved by the Ethics Committee of Qilu Hospital, Shandong University, Jinan, China.

General procedure

We have described the LELAPE procedure in a previous report[13]. The abdominal procedure was performed with the patient being placed in the Trendelenburg position, and the port placement was set up as shown in Figure 1A. After laparoscopic exploration, dissection and division of the pedicle of the inferior mesenteric vessels were performed. The sigmoid colon was mobilized from medial to lateral, and the rectum was mobilized following the total mesorectal excision principle. The sigmoid mesentery was trimmed at the rectosigmoid junction, where the rectum was transected with an endoscopic linear stapler (Figure 1B). The distal rectum and mesorectum were pushed down to the pelvic cavity (Figure 1C).

Modified primary closure

For modified primary closure, the pelvic peritoneum was closed with continuous suturing using a barbed suture (Covidien, Shanghai, China) (Figure 1D and E) before
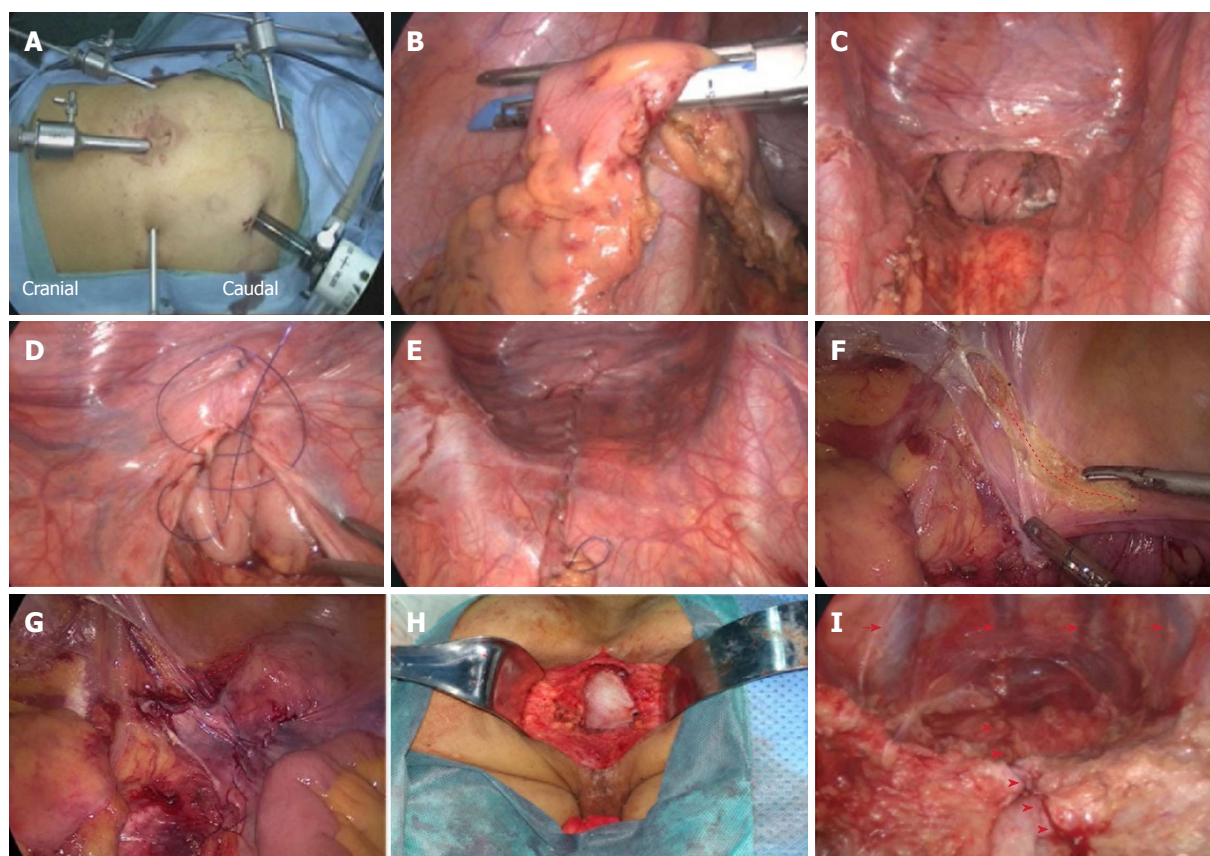
Figure 1 Surgical procedures. A: Port placement; B: Transection of the rectum at the rectosigmoid junction with an ENDO-GIA; C: Distal rectum pushed down to the pelvis; D: Closure of the pelvic peritoneum with a continuous suture using a barbed thread; E: Closure of the pelvic peritoneum; F: Tension reduction of the adjacent peritoneum (the dotted line shows the incised peritoneum); G: Closure of the peritoneum after tension reduction (the dotted line shows the incised peritoneum); H: Reconstruction of the pelvic floor with biological mesh; I: View of the closed peritoneum from the perineal wound in the prone position (the arrows show the presacral veins, and the arrowheads show the closed peritoneum).

creation of a colostomy. For tension-free suturing, the adjacent pelvic peritoneum was dissected to reduce tension if necessary (Figure 1F and G). The patient was turned over to the prone jackknife position. The levator ani was transected at its origin, and a cylindrical specimen was removed. The procedure was completed with both the placement of one negative-pressure drainage tube in the presacral space, and the layered closure of the ischiorectal fat and skin. The drainage tube was removed when the drainage fluid was clear and < 10 mL in volume. The coccyx was not routinely removed.

Biological mesh closure

For biological mesh closure, the patient was changed into a prone position for perineal dissection after creation of a colostomy. The levator ani was transected at its origin, and a cylindrical specimen was removed. A human acellular dermal matrix mesh (Ruinuo, Qingyuanweiye Bio-Tissue Engineering Ltd., Beijing, China) was implanted and fixed to the tendinous arch by continuous prolene sutures (Covidien) for reconstruction of the pelvic floor (Figure 1H). The procedure was completed with both the placement of one negative-pressure drainage tube below the mesh, and the layered closure of the ischiorectal fat and skin. All the operations were finished by the same surgical group.

Statistical analysis

Numerical data were expressed as mean $\pm$ SD and analyzed with Student's t tests. Categorical data were analyzed with the χ test or Fisher's exact test. Repeated measures analysis of variance was performed for postoperative drainage and temperature change. All analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, United States). $P < 0.05$ was considered to be statistically significant.

RESULTS

The baseline characteristics, including male/female ratio, age, body mass index, neoadjuvant therapy, distance to anal verge, and postoperative TNM staging, were comparable between the two groups ($P > 0.05$ each, Table 1). All patients were successfully followed up postoperatively for one year.

All operations were successfully performed without serious intraoperative complications. The pelvic reconstruction time was 14.6 $\pm$ 3.7 min for the modified primary closure group, which was significantly longer than that of the biological mesh closure group (7.2 $\pm$
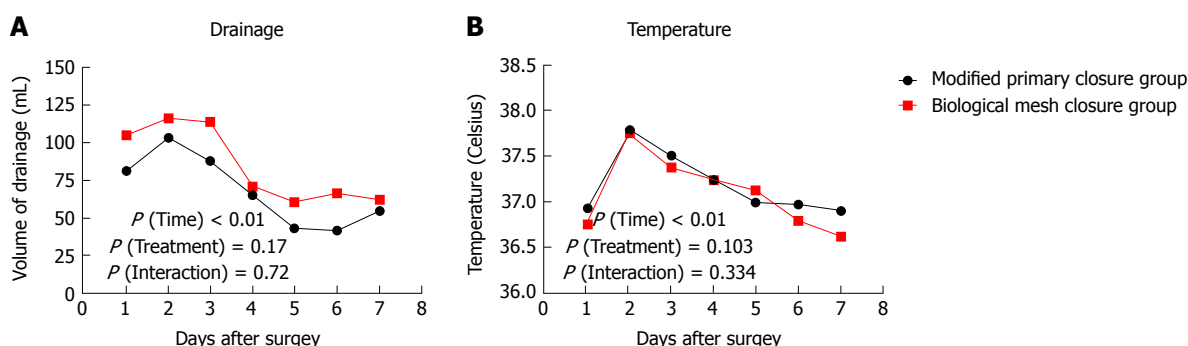
Table 1 Baseline characteristics.

	Modified primary closure (*n* = 32)	Biological mesh closure (*n* = 44)	*P* value
Male/female	24/8	31/13	0.662
Age (yr)	52.8 ± 12.2	58.2 ± 12.5	0.137
BMI	26.8 ± 3.2	25.7 ± 2.7	0.097
Neoadjuvant therapy	8	7	0.326
Tumor location, Distance to anal verge (cm)	2.6 ± 0.8	2.8 ± 0.9	0.278
Postoperative TNM staging			
Ⅱ	23	29	0.581
Ⅲ	9	15	

BMI: Body mass index.

Table 2 Perioperative data.

	Modified primary closure (*n* = 32)	Biological mesh closure (*n* = 44)	*P* value
Reconstruction time (min)	14.6 ± 3.7	7.2 ± 1.9	< 0.001
Total operative time (min)	236 ± 20	248 ± 43	0.143
Intraoperative blood loss (mL)	165 ± 57	149 ± 52	0.242
Positive CRM	0	0	N/A
Bowel perforation	0	1	1.000
Recovery of bowel function (h)	22.8 ± 4.7	23.6 ± 5.0	0.475
Intestinal obstruction	0	1	1.000
Drainage removal (days after surgery)	6.6± 1.1	7.3 ± 2.0	0.094
Postoperative hospital stay (d)	8.1 ± 1.9	10.1 ± 2.8	0.001
Cost (USD)	9297 ± 1260	10719 ± 2360	0.003

CRM: Circumferential margin; USD: United States Dollar.

Figure 2 Drainage and temperature changes. A: Postoperative drainage volumes in the two groups; B: Postoperative temperature changes in the two groups.

1.9 min, *P* < 0.001). The total operating time did not differ between the two groups (236 ± 20 min *vs* 248 ± 43 min, *P* = 0.143). One patient in the biological mesh closure group developed bowel perforation due to a large tumor within the anterior wall of the rectum. No positive circumferential margin was observed in either group. Intraoperative blood loss and recovery of bowel function were comparable between the two groups (both *P* > 0.05) (Table 2). The drainage tube was removed postoperatively at 6.6 ± 1.1 d in the modified primary closure group, which was earlier than in the biological mesh closure group (7.3 ± 2.0 d, *P* = 0.094). The volume of drainage fluid peaked at 2 d postoperatively and then decreased gradually, without any difference between the two groups (*P* treatment > 0.05, *P* interaction > 0.05)

(Figure 2A). The temperature changes after operation showed a similar pattern to drainage volume, with no difference between the groups (*P* treatment > 0.05, *P* interaction > 0.05) (Figure 2B). Postoperative hospital stay was 8.1 ± 1.9 d, and the total cost was 9297 ± 1260 USD for the modified primary closure group. Both of these categories in this group were significantly less than those of the biological mesh closure group (*P* = 0.001 and *P* = 0.003, respectively) (Table 2). One patient in the biological mesh closure group developed postoperative intestinal obstruction at 40 d. Conservative therapy did not work, and a laparoscopic exploration was performed at 42 d. The middle part of the ileum, approximately 100 cm to the ileocecal junction, adhered to the pelvic floor, leading to dilation of the proximal small intestine

Table 3 Follow-up data

	Modified primary closure (*n* = 32)	Biological mesh closure (*n* = 44)	*P* value
Normal perineal wound healing[1]			
10 d postoperatively	29	39	0.546
30 d postoperatively	27	41	0.270
60 d postoperatively	32	44	1.000
Perineal wound infection	5	5	0.734
Clear or haemoserous discharge	3	1	0.304
Pus/purulent discharge	1	2	1.000
Deep infection with or without tissue breakdown	1	2	1.000
Postoperative perineal hernia (12 mo)	0	0	N/A
Postoperative feeling of bulge (12 mo)	4	2	0.233
Postoperative chemotherapy	23	28	0.330
Postoperative radiotherapy	7	14	0.339
Postoperative local recurrence (12 mo)	0	1	1
Postoperative liver/lung metastasis (12 mo)	2	3	1
Postoperative death (12 mo)	0	0	N/A

[1]Grade 0 or Grade Ⅰ by the Southampton Wound Scoring System.

Figure 3 Laparoscopy. Laparoscopic exploration of the abdominal cavity in the patient with intestinal obstruction (the arrow shows the proximal dilated small intestine, and the arrowhead shows the distal normal small intestine).

(Figure 3). The patient was healed by decompression of the small intestine and intestinal rearrangement under laparotomy.

In the modified primary closure group, five patients had perineal wound infection. Within the first 10 d postoperatively, three patients had haemoserous discharge from the perineal wound, and were healed following potassium permanganate hip bath after 1 mo. At 12 d, one patient showed purulent discharge, which was solved after daily dressing change and thermal therapy. At 15 d, the perineal wound broke down in one female patient with type 2 diabetes. Debridement and secondary suturing were performed at 33 d after daily dressing change. Likewise, in the biological mesh closure group, five patients had perineal wound infection within the first 10 d (Table 3), and recovered within 60 d after appropriate treatment. No difference in infection rate or grade was found between the two groups (*P* > 0.05 each). Perineal hernia is theoretically expected to be more frequent without the placement of meshes. However, at 12 mo postoperatively, no perineal hernia occurred in either of the groups. Notably, four patients in the modified primary closure group and two in the

biological mesh group experienced the feeling of bulging. Computed tomography at 12 mo showed that in the modified primary closure group, the small intestine was kept in the pelvic cavity with a clear descent of the pelvic peritoneum. In the biological mesh group, without suturing the pelvic peritoneum, the small intestine was also kept in the presacral space. No obvious postoperative differences were detected in the computed tomography scans between the two groups at 12 mo (Figure 4).

Postoperative chemotherapy (the XELOX or FOLFOX regimen) and radiotherapy were given to 23 and seven patients in the modified primary closure group, as well as 28 and 14 patients in the biological mesh closure group, respectively (*P* = 0.330 and *P* = 0.339). In the biological mesh closure group, local recurrence occurred in one patient, who received only postoperative chemotherapy with the XELOX regimen, and the patient was subsequently treated with radiotherapy. Three patients had minor liver metastases and were cured with local ablative treatment. In the modified primary closure group, minor lung metastasis and minor liver metastases were found in two patients, respectively. Both of these
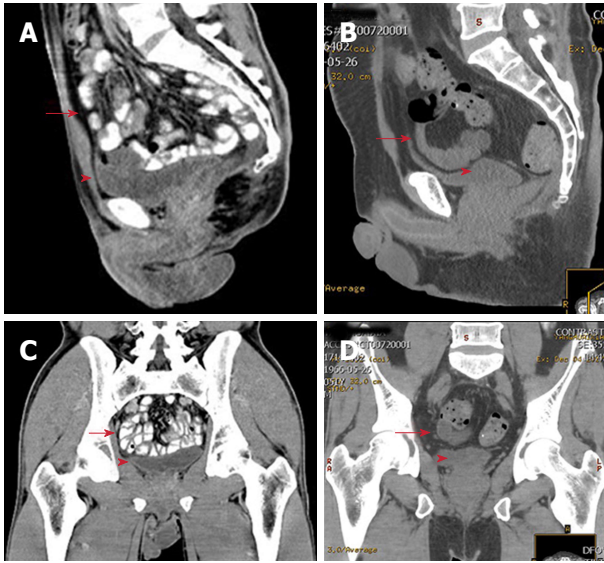
Figure 4 Postoperative magnetic resonance imaging. A: Twelve-month postoperative Sagittal CT scan in the modified primary closure group; B: Twelve-month postoperative Sagittal CT scan in the biological mesh closure group; C: Twelve-month postoperative Coronal CT scan in the modified primary closure group; D: Twelve-month postoperative Coronal CT scan in the biological mesh closure group (the arrow shows the small intestine, and the arrowhead shows the bladder). CT: Computed tomography.

patients received local ablative treatment. No patients died in either of the two groups.

DISCUSSION

The necessity of reconstruction of the pelvic floor after ELAPE has been widely accepted in order to avoid postoperative perineal complications[14,15]. However, the feasibility and superiority of various methods proposed for this reconstruction remain to be investigated. In the present study, we compared two methods, modified primary closure and biological mesh closure, in 76 patients with lower rectal cancer undergoing LELAPE. The major findings were that modified primary closure required longer reconstruction time, shorter postoperative hospital stay and was more cost-effective when compared to biological mesh closure. No difference in other perioperative data, long-term complications or oncological outcomes was observed.

Various methods have been developed for perineal wound healing after ELAPE. Of these, perineal closure with myocutaneous flaps, biological or synthetic mesh placement, and omentoplasty with perineal closure are currently the most widely performed[8-12]. Myocutaneous flaps can be obtained by various approaches, including gluteal rotation/advancement flaps[16], inferior gluteal artery myocutaneous island transposition flaps (IGAM)[17], transverse rectus/vertical rectus abdominis (TRAM/VRAM)[18,19], and gracilis[20]. Myocutaneous flaps have the benefit of delivering good perfusion and oxygenation, thus facilitating the healing process of large perineal defects. However, this approach requires plastic surgeons and may cause additional complications (*e.g.*, a donor

site hernia)[14]. Mesh repair has the advantage of reducing operative duration, and is therefore more cost-effective compared to myocutaneous flaps[21]. However, it should be noted that the inertness of biomesh might be a reason for small bowel obstruction[22], and synthetic mesh carries the potential for fistula formation[23]. By contrast, omentoplasty with perineal closure represents a safer approach for the reconstruction of the pelvic floor. Owing to its rich lymphovascular supply, the mobilized omentum in the pelvic cavity inhibits regional fluid collection, and hence prevents small intestine adhesion to the pelvic floor, thus dramatically reducing related complications[24]. For some patients, this technique may not apply when it is not technically feasible to mobilize the omentum to reach the pelvic cavity, or when the omentum has been resected previously.

The major strength of the modified primary closure method is the reconstruction of the pelvic peritoneum, which keeps the small intestine in the abdominal and pelvic cavities, thus avoiding adhesion to extraperitoneal tissues. In the present study, one case of intestinal obstruction in the biological mesh closure group appeared, which was caused by adhesion of the small intestine to the pelvic floor. However, the rate of postoperative intestinal obstruction did not show any difference between the two groups, which is likely due to insufficient study power. Compared with biological mesh closure, modified primary closure reduced postoperative hospital stay duration and total cost.

The pelvic floor is usually left open after APR due to the concern that incomplete closure of the pelvic floor may cause pelvic floor hernias and intestinal obstruction. However, APR is associated with clinically significant perineal hernias, albeit < 1% of the incidence[23]. ELAPE requires extensive resection of the pelvic floor, and thus contributes to the development of perineal hernias, with an incidence of 2.8% *vs* 0.8% compared to traditional APR[1]. As to LELAPE, perineal hernias could occur in nearly half of the patients without reconstruction of the pelvic floor[25]. In LELAPE, closure of the pelvic peritoneum is more challenging because the distal rectum has not been removed at that time. When mobilizing the sigmoid and rectum, the peritoneum on both sides should be intentionally preserved for re-approximation of the pelvic peritoneum. One possible concern is that the intentionally preserved peritoneum may lead to compromised oncological outcomes. However, in the present study, the oncological outcomes did not show any difference between the two groups. The rectum should be transected at the sigmoidorectal junction area, or even lower if possible. A continuous suture with barbed thread is recommended to facilitate the procedure. In obese patients, the peripheral peritoneum should be dissected to reduce tension. The perineal wound was directly sutured in layers. No pelvic floor hernias and perineal hernias occurred in all of our patients, with a mean followup of 12 mo. With regard to patients with rigid peritoneums after neoadjuvant radiotherapy or large pelvic peritoneum defects, this procedure may not

be eligible and other reconstructive methods should be applied.

In conclusion, based on our preliminary experience, the modified primary closure method for reconstruction of the pelvic floor is technically feasible, safe and cost-effective. However, as the present study was retrospective, the safety and feasibility of this method still warrants high evidence-level research.

ARTICLE HIGHLIGHTS

Research background
Laparoscopic extralevator abdominal perineal excision (LELAPE) was introduced to reduce the rate of positive circumferential margins and intraoperative perforation, however its extensive dissection requires reconstruction of the pelvic floor.

Research motivation
To introduce a novel modified primary closure technique of LELAPE for low rectal cancer.

Research objectives
To assess the feasibility, safety and cost-effectiveness of the newly introduced technique by comparing it with the traditional method.

Research methods
Data from 76 patients with rectal cancer undergoing LELAPE from March 2013 to May 2016 were retrospectively analyzed. Patients were classified into the modified primary closure group (32 patients) and the biological mesh closure group (44 patients). Total operating time, reconstruction time, postoperative stay duration, total cost, postoperative complications and tumor recurrence were compared.

Research results
The modified primary closure of the pelvic floor requires longer reconstruction time, but total operating time was not different compared with the biological mesh closure group. The postoperative length of hospital stay and the total cost were both less in the modified primary closure group. No differences in other perioperative data, long-term complications or oncological outcomes were observed.

Research conclusions
The modified primary closure method for reconstruction of the pelvic floor in LELAPE for low rectal cancer is technically feasible, safe and cost-effective.

Research perspectives
Future multicentered randomized controlled trials should be performed to confirm the conclusions made in the present study.

REFERENCES

1   **West NP**, Anderin C, Smith KJ, Holm T, Quirke P; European Extralevator Abdominoperineal Excision Study Group. Multicentre experience with extralevator abdominoperineal excision for low rectal cancer. *Br J Surg* 2010; **97**: 588-599 [PMID: 20186891 DOI: 10.1002/bjs.6916]

2   **Yu HC**, Peng H, He XS, Zhao RS. Comparison of short- and long-term outcomes after extralevator abdominoperineal excision and standard abdominoperineal excision for rectal cancer: a systematic review and meta-analysis. *Int J Colorectal Dis* 2014; **29**: 183-191 [PMID: 24271080 DOI: 10.1007/s00384-013-1793-7]

3   **Yang Y**, Xu H, Shang Z, Chen S, Chen F, Deng Q, Luo L, Zhu L, Shi B. Outcome of extralevator abdominoperineal excision over conventional abdominoperineal excision for low rectal tumor: a meta-analysis. *Int J*

Clin Exp Med 2015; **8**: 14855-14862 [PMID: 26628967]

4   **Stelzner S**, Hellmich G, Sims A, Kittner T, Puffer E, Zimmer J, Bleyl D, Witzigmann H. Long-term outcome of extralevator abdominoperineal excision (ELAPE) for low rectal cancer. *Int J Colorectal Dis* 2016; **31**: 1729-1737 [PMID: 27631643 DOI: 10.1007/s00384-016-2637-z]

5   **Chi P**, Chen ZF, Lin HM, Lu XR, Huang Y. Laparoscopic extralevator abdominoperineal resection for rectal carcinoma with transabdominal levator transection. *Ann Surg Oncol* 2013; **20**: 1560-1566 [PMID: 23054115 DOI: 10.1245/s10434-012-2675-x]

6   **Kipling SL**, Young K, Foster JD, Smart NJ, Hunter AE, Cooper E, Francis NK. Laparoscopic extralevator abdominoperineal excision of the rectum: short-term outcomes of a prospective case series. *Tech Coloproctol* 2014; **18**: 445-451 [PMID: 24081545 DOI: 10.1007/s10151-013-1071-2]

7   **Christensen HK**, Nerstrøm P, Tei T, Laurberg S. Perineal repair after extralevator abdominoperineal excision for low rectal cancer. *Dis Colon Rectum* 2011; **54**: 711-717 [PMID: 21552056 DOI: 10.1007/DCR.0b013e3182163c89]

8   **Musters GD**, Buskens CJ, Bemelman WA, Tanis PJ. Perineal wound healing after abdominoperineal resection for rectal cancer: a systematic review and meta-analysis. *Dis Colon Rectum* 2014; **57**: 1129-1139 [PMID: 25101610 DOI: 10.1097/DCR.0000000000000182]

9   **Peirce C**, Martin S. Management of the Perineal Defect after Abdominoperineal Excision. *Clin Colon Rectal Surg* 2016; **29**: 160-167 [PMID: 27247542 DOI: 10.1055/s-0036-1580627]

10 **Sumrien H**, Newman P, Burt C, McCarthy K, Dixon A, Pullyblank A, Lyons A. The use of a negative pressure wound management system in perineal wound closure after extralevator abdominoperineal excision (ELAPE) for low rectal cancer. *Tech Coloproctol* 2016; **20**: 627-631 [PMID: 27380256 DOI: 10.1007/s10151-016-1495-6]

11 **Alam NN**, Narang SK, Köckerling F, Daniels IR, Smart NJ. Biologic Mesh Reconstruction of the Pelvic Floor after Extralevator Abdominoperineal Excision: A Systematic Review. *Front Surg* 2016; **3**: 9 [PMID: 26909352 DOI: 10.3389/fsurg.2016.00009]

12 **Ge W**, Jiang SS, Qi W, Chen H, Zheng LM, Chen G. Extralevator abdominoperineal excision for rectal cancer with biological mesh for pelvic floor reconstruction. *Oncotarget* 2017; **8**: 8818-8824 [PMID: 27732566 DOI: 10.18632/oncotarget.12502]

13 **Wang YL**, Dai Y, Jiang JB, Yuan HY, Hu SY. Application of laparoscopic extralevator abdominoperineal excision in locally advanced low rectal cancer. *Chin Med J* (Engl) 2015; **128**: 1340-1345 [PMID: 25963355 DOI: 10.4103/0366-6999.156779]

14 **Butt HZ**, Salem MK, Vijaynagar B, Chaudhri S, Singh B. Perineal reconstruction after extra-levator abdominoperineal excision (eLAPE): a systematic review. *Int J Colorectal Dis* 2013; **28**: 1459-1468 [PMID: 23440362 DOI: 10.1007/s00384-013-1660-6]

15 **Jensen KK**, Rashid L, Pilsgaard B, Møller P, Wille-Jørgensen P. Pelvic floor reconstruction with a biological mesh after extralevator abdominoperineal excision leads to few perineal hernias and acceptable wound complication rates with minor movement limitations: single-centre experience including clinical examination and interview. *Colorectal Dis* 2014; **16**: 192-197 [PMID: 24251666 DOI: 10.1111/codi.12492]

16 **Anderin C**, Martling A, Lagergren J, Ljung A, Holm T. Short-term outcome after gluteus maximus myocutaneous flap reconstruction of the pelvic floor following extra-levator abdominoperineal excision of the rectum. *Colorectal Dis* 2012; **14**: 1060-1064 [PMID: 21981319 DOI: 10.1111/j.1463-1318.2011.02848.x]

17 **Boccola MA**, Rozen WM, Ek EW, Grinsell D, Croxford MA. Reconstruction of the irradiated extended abdominoperineal excision (APE) defect for locally advanced colorectal cancer. *J Gastrointest Cancer* 2011; **42**: 26-33 [PMID: 20972664 DOI: 10.1007/s12029-010-9224-2]

18 **Petrie N**, Branagan G, McGuiness C, McGee S, Fuller C, Chave H. Reconstruction of the perineum following anorectal cancer excision. *Int J Colorectal Dis* 2009; **24**: 97-104 [PMID: 18688618 DOI: 10.1007/s00384-008-0557-2]

19 **McMenamin DM**, Clements D, Edwards TJ, Fitton AR, Douie WJ. Rectus abdominis myocutaneous flaps for perineal reconstruction:

modifications to the technique based on a large single-centre experience. *Ann R Coll Surg Engl* 2011; **93**: 375-381 [PMID: 21943461 DOI: 10.1308/003588411X572268]

20   **Shibata D**, Hyland W, Busse P, Kim HK, Sentovich SM, Steele G Jr, Bleday R. Immediate reconstruction of the perineal wound with gracilis muscle flaps following abdominoperineal resection and intraoperative radiation therapy for recurrent carcinoma of the rectum. *Ann Surg Oncol* 1999; **6**: 33-37 [PMID: 10030413 DOI：10.1007/s10434-999-0033-4]

21   **Peacock O**, Pandya H, Sharp T, Hurst NG, Speake WJ, Tierney GM, Lund JN. Biological mesh reconstruction of perineal wounds following enhanced abdominoperineal excision of rectum (APER). *Int J Colorectal Dis* 2012; **27**: 475-482 [PMID: 22006494 DOI: 10.1007/s00384-011-1325-2]

22   **Jess P**, Bulut O. Small bowel obstruction after reconstruction of the pelvic floor with porcine dermal collagen (Permacol) after extended abdominoperineal extirpation for rectal cancer: report of two cases. *Colorectal Dis* 2010; **12**: e178-e179 [PMID: 19832869 DOI: 10.1111/j.1463-1318.2009.02060.x]

23   **Mjoli M**, Sloothaak DA, Buskens CJ, Bemelman WA, Tanis PJ. Perineal hernia repair after abdominoperineal resection: a pooled analysis. *Colorectal Dis* 2012; **14**: e400-e406 [PMID: 22308975 DOI: 10.1111/j.1463-1318.2012.02970.x]

24   **Hultman CS**, Sherrill MA, Halvorson EG, Lee CN, Boggess JF, Meyers MO, Calvo BA, Kim HJ. Utility of the omentum in pelvic floor reconstruction following resection of anorectal malignancy: patient selection, technical caveats, and clinical outcomes. *Ann Plast Surg* 2010; **64**: 559-562 [PMID: 20395804 DOI: 10.1097/SAP.0b013e3181ce3947]

25   **Sayers AE**, Patel RK, Hunter IA. Perineal hernia formation following extralevator abdominoperineal excision. *Colorectal Dis* 2015; **17**: 351-355 [PMID: 25413255 DOI: 10.1111/codi.12843]

ORIGINAL ARTICLE

Observational Study

Altered oral microbiota in chronic hepatitis B patients with different tongue coatings

Yu Zhao, Yu-Feng Mao, Yi-Shuang Tang, Ming-Zhu Ni, Qiao-Hong Liu, Yan Wang, Qin Feng, Jing-Hua Peng, Yi-Yang Hu

Yu Zhao, Yu-Feng Mao, Yi-Shuang Tang, Ming-Zhu Ni, Qiao-Hong Liu, Yan Wang, Qin Feng, Jing-Hua Peng, Institute of Liver Diseases, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

Yi-Yang Hu, Institute of Liver Diseases, Key Laboratory of Liver and Kidney Diseases (Ministry of Education), Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

ORCID number: Yu Zhao (0000-0002-2208-2882); Yu-Feng Mao (0000-0002-8604-9096); Yi-Shuang Tang (0000-0001-5125-8376); Ming-Zhu Ni (0000-0002-5675-953X); Qiao-Hong Liu (0000-0001-9887-8126); Yan Wang (0000-0002-6710-8695); Qin Feng (0000-0002-4641-1636); Jing-Hua Peng (0000-0002-0665-7666); Yi-Yang Hu (0000-0001-9127-7002).

Author contributions: Hu YY designed the study; Zhao Y and Tang YS performed the tongue coating microbial experiments; Tang YS, Liu QH and Wang Y performed the tongue coating metabolic experiments; Tang YS, Feng Q and Peng JH performed the clinical trial; Mao YF and Ni MZ performed the statistical analyses; Zhao Y wrote the paper.

Supported by the Shanghai Educational Development Foundation, No. 14CG41; the National Natural Science Foundation of China, No. 81403298 and No. 81373857; and the National Key New Drug Creation Project, No. 2017ZX09304002.

Institutional review board statement: The study was reviewed and approved by the IRB of Shuguang Hospital affiliated with the Shanghai University of TCM Institutional Review Board.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: There are no conflicts of interest to report.

STROBE statement: The authors have read the STROBE Statement checklist of items, and the manuscript was prepared and revised according to the STROBE Statement checklist of items.

Abstract

AIM

To elucidate tongue coating microbiota and metabolic differences in chronic hepatitis B (CHB) patients with yellow or white tongue coatings.

METHODS

Tongue coating samples were collected from 53 CHB

patients (28 CHB yellow tongue coating patients and 25 CHB white tongue coating patients) and 22 healthy controls. Microbial DNA was extracted from the tongue samples, and the bacterial 16S ribosomal RNA gene V3 region was amplified from all samples and sequenced with the Ion Torrent PGM™ sequencing platform according to the standard protocols. The metabolites in the tongue coatings were evaluated using a liquid chromatography-mass spectrometry (LC-MS) platform. Statistical analyses were then performed.

RESULTS
The relative compositions of the tongue coating microbiotas and metabolites in the CHB patients were significantly different from those of the healthy controls, but the tongue coating microbiota abundances and diversity levels were not significantly different. Compared with the CHB white tongue coating patients, the CHB yellow tongue coating patients had higher hepatitis B viral DNA (HBV-DNA) titers (median 21210 *vs* 500, respectively, P = 0.03) and a significantly lower level of Bacteroidetes (20.14% *vs* 27.93%, respectively, P = 0.013) and higher level of Proteobacteria (25.99% *vs* 18.17%, respectively, P = 0.045) in the microbial compositions at the phylum level. The inferred metagenomic pathways enriched in the CHB yellow tongue coating patients were mainly those involved in amino acid metabolism, which was consistent with the metabolic disorder. The abundances of bacteria from Bacteroidales at the order level were higher in the CHB white tongue coating patients (19.2% *vs* 27.22%, respectively, P = 0.011), whereas Neisseriales were enriched in the yellow tongue coating patients (21.85% *vs* 13.83%, respectively, P = 0.029). At the family level, the abundance of Neisseriaceae in the yellow tongue patients was positively correlated with the HBV-DNA level but negatively correlated with the S-adenosyl-L-methionine level.

CONCLUSION
This research illustrates specific clinical features and bacterial structures in CHB patients with different tongue coatings, which facilitates understanding of the traditional tongue diagnosis.

Key words: 16S rRNA gene sequencing; Metabolomics; Chronic hepatitis B; Tongue diagnosis; Microbiota

Core tip: Tongue diagnosis has important guiding significance for clinical syndrome differentiation and drug use in traditional Chinese medicine (TCM), but lacks scientific explanations. This study illustrates the existence of specific clinical features and bacterial structure in chronic hepatitis B (CHB) patients with different tongue coatings. Compared with the CHB white tongue coating patients, the yellow tongue coating patients had higher viral titers and a significantly lower level of Bacteroidetes and higher level of Proteobacteria in their microbial compositions at the phylum level. This study explores the micro-features between different tongue coatings, which will promote our understanding of the TCM tongue diagnosis and facilitate therapeutic strategies for individualized treatment.

INTRODUCTION
Tongue diagnosis is a characteristic diagnostic method of traditional Chinese medicine (TCM) with a long history. TCM believes that the tongue is an important window for changes in the body, which are closely related to the *zang* and *fu* (internal organs), meridians, *qi* and *xue* (blood) and *body fluids*. Therefore, changes of the tongue can help doctors distinguish syndromes and guide diagnoses, which has great significance for clinical treatment *via* TCM. Different tongue coating colors in TCM have different treatment principles. Tongue diagnosis has the advantages of convenience and intuitiveness and represents first-hand information for TCM doctors that should not neglected. However, the physiological and pathological changes that contribute to the TCM tongue diagnosis have not been elucidated. According to TCM theory, the formation of tongue coating is closely related to *wei-qi* (spleen and stomach). Reports have noted that micro-ecological changes in the local flora are important manifestations of pathological tongue images[1]. As part of the human microbiome, the oral microbiota can interfere with the gut microbiota and is associated with multiple diseases[2]. The microbiota can produce metabolites and active ingredients that participate in the regulation of host metabolism and immunity and is closely related to the pathological processes of many diseases. Some reports have found abnormalities in the microbiota or metabolites in pathological tongue coatings[3,4]. However, integrated analyses of the pathological tongue coating microbiotas and metabolites have rarely been reported. As an important diagnostic factor, elucidating the "intension" of the material basis of tongue coatings and providing the scientific basis for individualized treatment and clinical syndrome differentiation have great significance in TCM.

Chronic hepatitis B (CHB) is a major infectious disease that may lead to liver cirrhosis and hepatocellular carcinoma (HCC). In China, CHB is a huge health burden, and 60% and 80% of China's liver cirrhosis and HCC patients, respectively, are infected with hepatitis B virus (HBV)[5]. Intestinal bacteria are widely involved in the pathogenesis of chronic liver diseases, which can affect liver homeostasis based on energy absorption and storage, interfere with bile metabolism, participate in immune regulation and affect other processes[6].

Some studies have shown that dysbiosis of the oral microbiota may be involved in the development of HBV-induced chronic liver disease. Moreover, key oral-derived phylotypes may invade the gut as opportunistic pathogens and alter the composition of the gut microbiota, and cirrhotic patients have a more limited salivary defenses and worse inflammation than healthy patients[7]. In China, many patients with chronic liver disease are treated with TCM, which has been reported to decrease the incidence of liver cancer in a recent 15-year follow-up study of 21020 newly diagnosed CHB patients[8]. Changes in the tongue are important for TCM diagnoses[9,10], and CHB patients have different tongue coating phenotypes, which most commonly manifested as a yellow or a white tongue coating. These tongue coatings lead to different treatment strategies in TCM. Therefore, we speculate that microbiota differences may exist between CHB patients with different tongue coatings.

In this study, we characterize the microbiota and metabolic compositions of CHB patients with yellow or white tongue coatings and investigate the association between the tongue coating microbiotas, metabolites and host physiological indices to elucidate the differences between CHB patients with yellow and white tongue coatings. These results will contribute to our understanding of the relationship between the tongue coating appearance and the microbiota and metabolic micro-features differences.

MATERIALS AND METHODS

Patients
All samples from this study were collected at the Shanghai University of TCM-affiliated Shuguang Hospital in Shanghai, the Nanjing Second Hospital and the Huai'an Fourth People's Hospital in Jiangsu province, People's Republic of China, from 2013 to 2014. The criteria used for the diagnosis of CHB were from the "The Guidelines for the Prevention and Treatment of Chronic Hepatitis B (2010 version)" issued by the Chinese Society of Liver Diseases and the Chinese Society of Infectious Diseases. Healthy age- and gender-matched control subjects were recruited from the Shanghai University of TCM-affiliated Shuguang Hospital Medical Center. We excluded patients who had been on absorbable antibiotics within the past 3 mo; were undergoing periodontal disease treatment; had other viral liver hepatitises, liver cirrhosis, or HCC; were pregnant or lactating women; or had other severe primary diseases. Informed written consent was obtained before the participants' enrollment. This project was approved by the IRB of Shuguang Hospital, which was affiliated with the Shanghai University of TCM (Permit Number: 2012-206-22-01) and conformed to the ethical guidelines of the Declaration of Helsinki (2008).

Sample collection
Each subject underwent serum and tongue coating collection on the same day in the morning before breakfast. For tongue coating collection, all participants were required to rinse their mouth with physiological saline and then were photographed with the tongue diagnostic information acquisition system in a stable light source (Daosh Co., Shanghai, China). Sterile spoons scraped the fixed parts of the tongue surface twice to collect tongue coating samples, which were dissolved in sanitized Eppendorf tubes filled with 2 mL of physiological saline[4]. Spare samples were stored in a -80 ℃ freezer. Blood samples were taken for clinical analyses after an overnight fast of at least 10 h.

Biochemical measurements
Clinical and biochemical indices were determined for each patient, including the following: (1) liver and kidney function, including serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), total bilirubin (TBIL), direct bilirubin (DBIL), albumin (ALB), globulin (GLB), creatinine (Cr), blood urea nitrogen (BUN) and uric acid (UA); (2) lipid profiles, including triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL) and low-density lipoprotein (LDL); (3) clotting function, including the prothrombin time (PT); and (4) virological indicators, including hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (HBsAb), hepatitis Be antigen (HBeAg), hepatitis Be antibody (HBeAb), hepatitis B core antibody (HBcAb) and the HBV-DNA titers were determined for each patient.

Tongue coating microbial diversity analysis
DNA extraction and PCR amplification: Microbial DNA was extracted from the tongue samples using a QIAGEN QIAamp DNA Stool Mini Kit (50) according to manufacturer's protocols. The V3 region of the bacterial 16S ribosomal RNA (rRNA) gene was amplified by PCR (95 ℃ for 2 min, followed by 25 cycles at 95 ℃ for 30 s, 55 ℃ for 30 s and 72 ℃ for 30 s and a final extension at 72 ℃ for 5 min) using the primers 341F (5'-CCTACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3'), which included a barcode that was an eight-base sequence unique to each sample. The PCR reactions were performed in triplicate with a 20 μL mixture containing 4 μL of 5 × FastPfu buffer, 2 μL of 2.5 mmol/L dNTPs, 0.8 μL of each primer (5 μmol/L), 0.4 μL of KAPA HiFi Polymerase, and 10 ng of template DNA.

Ion Torrent PGM™ sequencing: Amplicons were extracted from 2% agarose gels, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to the manufacturer's instructions and then quantified using the Qubit 2.0 Fluorometer (Invitrogen, United States). Purified amplicons were pooled at equimolar concentrations and sequenced on an Ion Torrent PGM™ platform according to standard protocols. The raw reads were deposited

into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP127002).

Processing of sequencing data: Raw FASTQ files were demultiplexed and quality-filtered using the fastx_Toolkit (version 0.0.13.2) with the following criteria. The 200 bp reads were truncated at any site receiving an average quality score < 20, and truncated reads that were shorter than 50 bp were discarded. Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UCHIME (version 7.1 http://drive5.com/uparse/), with also identified and removed chimeric sequences. The taxonomy of each 16S rRNA gene sequence was analyzed using the RDP Classifier (http://rdp.cme.msu.edu/) against the SILVA (SSU123)16S rRNA database with a confidence threshold of 70%[11].

Metabolomic profiling of human tongue coating samples
Sample preparations: The tongue coating samples were thawed on ice, and quality control (QC) samples were made by mixing and blending equal volumes (20 μL) of each sample. A 100 μL aliquot of the samples was added to 500 μL of 100% methanol, which was vibrated for 30 s and centrifuged at 15000 *g* for 10 min. The supernatant was dried in a vacuum centrifuge at 30 ℃. The dried samples were dissolved in 300 μL of water/methanol (1:1, 4 ℃) and filtered through a 0.22 μm membrane. A 3 μL aliquot of tongue coating was injected for LC-MS analysis, and another 100 μL was sampled for subsequent MS/MS identification.

LC-MS experiments: A Waters ACQUITY UPLC system analysis was performed on an ACQUITY UPLC® HSS T3 column (150 mm × 2.1 mm, 1.8 μm particle size; Waters Corporation, Milford, MA, United States) for separation of samples using a binary gradient mode. The sample was eluted with a gradient mobile phase composed of 0.1% (v/v) formic acid/water (A) and 0.1% (v/v) formic acid/acetonitrile (B) as follows: 0-1 min, 2% B; 1-11 min, linear increase from 2 to 50% B; 11-17 min, linear increase from 50 to 98% B; 17-18 min, 98% B; 18-19 min, linear decrease from 98 to 2% B and 19-20 min, 2% B. The flow rate was set to 0.3 mL/min. The column and sample-tray temperatures were maintained at 35 and 8 ℃, respectively.

A Thermo Scientific™ LTQ XL™ mass spectrometer (Thermo Scientific, San Jose, CA, United States) was combined with electrospray ionization (ESI) operating in both the negative and positive ion modes. The positive ionization mode was operated using a spray voltage of 4.8 kV, and the negative ionization mode used a spray voltage of 4.50 kV. The capillary temperature was 325 ℃, and the sheath gas and auxiliary gas were set at flow rates of 45 and 10 L/min, respectively. The mass scanning range was set at 89-1000 m/z in positive ionization mode and 87-1000 m/z in negative ionization mode.

Data processing and metabolite identification: The raw LC-MS data files were converted into the mzXML format and then analyzed by the XCMS program in the R statistical language (v3.1.1) for peak identification, filtering and alignment. For the subsequent multivariate linear regression analysis, the XCMS results were ultimately converted into two-dimensional data matrices that consisted of retention time (Rt)-m/z pairs and their peak area. The data were normalized by their sum peak areas, and 1043 positive ion mode variables and 540 negative ion mode variables were finally obtained for the subsequent analysis.

Metabolites driving the differences among the 3 groups were filtered with a variable importance (VIP) > 1, a correlation P(corr) > 0.4, and a threshold of 1 using the 7-fold cross-validated partial least squares-discriminant analysis (PLS-DA) model. By using retention time-m/z pairs as the identifiers for each ion, we examined and optimized the parameters individually. The possible identities of the metabolites were first confirmed based on their exact molecular weights (molecular weight error < 15 ppm), followed by the comparison of their exact molecular weight and MS/MS fragmentation patterns to entries in the Human Metabolome Database (HMDB) (http://www.hmdb.ca) and the METLIN (https://metlin.scripps.edu), MassBank (http://www.massbank.jp) and mzCloud (https://www.mzcloud.org) databases.

Statistical analysis
We compared the clinical difference among the 3 groups using the Kruskal-Wallis test and the Nemenyi test was used for post-pairwise comparisons. The HBV-DNA titers between the CHB yellow and white tongue coating patients and the metabolites differences between the CHB and the healthy controls were compared using the standard non-parametric Mann-Whitney *U* test. Unsupervised principal component analysis (PCA) and supervised PLS-DA analysis were performed using the Simca-P software (version 13.0, Umetrics, Umea, Sweden). The microbiota results were analyzed using Metastats analysis, the Kruskal-Wallis test and the linear discriminant analysis (LDA) effect size (LEfSe) method was implemented in LEfSe v1.0[12]. The functionality of the microbiota was assessed using PiCRUST[13] and compared among the groups. The metabolomics enrichment analysis was performed using MetaboAnalyst 3.0[14] (http://www.metaboanalyst.ca). We correlated the microbiota with clinical index and metabolites and visualized the results with a Spearman's correlation coefficient> 0.3 and *P* < 0.05 in Cytoscape (version 3.6.0).

RESULTS

Physiological characteristics
A total of 75 subjects, including 22 healthy subjects and 53 CHB patients, were enrolled in this study. Twenty-eight of the 53 CHB patients exhibited a yellow tongue coating, and 25 CHB patients exhibited a white tongue coating; their physiological characteristics are shown in Table 1. The ages, genders and body mass indices (BMIs) did not significantly differ among the groups. The CHB group had a significantly higher level of liver function,

Table 1 Physiological characteristics of the subjects

| | Healthy | CHB patients | | *P* value |
		White tongue coating	Yellow tongue coating	
Age	34.09 ± 7.16	34.46 ± 9.92	38.08 ± 9.89	0.255
Gender, male/female	13/9	21/7	17/8	0.488
BMI (kg/m²)	21.22 (16.61-25.56)	23.44 (18.42-31.64)	23.30 (18.37-45.67)	0.077
Antiviral drugs (%)	/	78.60%	64%	0.24
Chinese herbs (%)	/	17.90%	28%	0.378
TBIL (μmol/L)	14.87 (8.29-31.62)	12.85 (7.9-27.7)	17.27 (6.4-45.6)	0.27
DBIL (μmol/L)	3.065 (1.7-6.76)	3.9 (1.64-9.2)	4.8 (2.05-34.7)[b]	0.006
IDBIL (μmol/L)	11.6 (6.3-25.7)	8.95 (5-18.7)	10.2 (4.2-19.6)	0.282
ALT (IU/L)	15 (10-34)	28.75 (8.5-170)[b]	47 (11.1-533)[c]	< 0.001
AST (IU/L)	17.5 (11-31)	27 (12-133)[b]	38 (14.8-543)[c]	< 0.001
ALP(IU/L)	15.28 (9-52)	25.8 (10.01-98)	38 (8.6-274)[c]	< 0.001
GGT (IU/L)	70.5 (35-119)	72.7 (37.1-131)	76.3 (41.7-202)	0.62
ALB (g/L)	44.97 (40.89-50.77)	48.7 (43.1-56.3)[ad]	46 (36.6-50.1)	0.003
PT (s)	12.55 (11.3-13.9)	12.7 (11.1-16.4)	13.3 (11.3-16)	0.032
HBV-DNA (IU/mL)	/	500 (500-44830000)[d]	21210 (500-324100000)	0.03
BUN (mmol/L)	4.39 (2.97-6.39)	4.96 (2.96-8.9)	4.2 (2.4-7.28)	0.074
Gr (μmol/L)	72.355 (45.1-106.67)	70 (27.4-96.7)	69 (35.9-83)	0.449
UN (μmol/L)	305.5 (201-406)	325 (207-550)	294 (154-429)	0.204
FPG (mmol/L)	5.2 (3.96-5.63)	5.2 (4.29-7.09)	5.215 (4.07-15.54)	0.532
TC (mmol/L)	4.04 (0.37-6.34)	4.55 (3.15-6.52)	4.36 (3.1-6.74)	0.102
TG (mmol/L)	0.84 (0.37-1.91)	1.17 (0.58-3.31)[a]	1.11 (0.68-2.62)[a]	0.008
HDL (mmol/L)	1.14 (0.74-1.76)	1.145 (0.72-1.86)	1.07 (0.58-2.02)	0.779
LDL (mmol/L)	2.205 (1.38-5.04)	2.27 (1.47-3.8)	2.44 (1.12-3.73)	0.647
APOA-A (g/L)	1.175 (0.85-1.62)	1.08 (0.76-1.91)[c]	1.02 (0.71-1.42)[c]	0.013

Quantitative results are expressed as median with minimum and maximum into brackets. The *P* values indicate the significance of difference among groups, and the Kruskal-Wallis test for 3 groups and Nemenyi test were used for post pairwise comparisons. The HBV-DNA titers between the chronic hepatitis B (CHB) yellow and white tongue coating patients were compared using the standard non-parametric Mann-Whitney *U* test. The antiviral drugs usage and the Chinese herbs usage were compared between CHB yellow and white tongue coating patients using the Chi-square test. [a]*P* < 0.05 *vs* healthy controls, [b]*P* < 0.01 *vs* healthy controls, [c]*P* < 0.001 *vs* healthy controls, [d]*P* < 0.05 *vs* CHB yellow tongue coating patients.

including ALT, AST and GGT activity. The CHB yellow tongue coating patients had a trend toward higher TBIL, DBIL, ALT, AST, ALP, GGT and PT levels. Additionally, the HBV viral titer was significantly higher in yellow tongue coating patients than in the white coating patients (*P* = 0.03), whereas the ALB level was lower in the yellow tongue coating patients (*P* = 0.003).

Overview of tongue coating microbial differences among the patients

In this study, the qualified sequences were rarefied to 9300 sequences per sample for downstream analysis. A summary is shown in Supplementary Table 1, Good's coverage values were high in the three groups, indicating that the sequencing depth was sufficient for investigation. As shown in Figure 1A show, the oral microbiota abundance and diversity level did not significantly differ between the CHB patients and the healthy controls. A PCA plot based on the OTU distributions showed that the overall compositions of the tongue coating microbiota were not significantly shifted by age, gender, BMI and antiviral treatment (Supplementary Figure 1). However, the PLS-DA plot showed a clear separation between the CHB patients and the healthy groups, and the CHB yellow tongue coating and white tongue coating patients were also well separated (Figure 1B). A Venn diagram showed that 1569 OTUs were common in all samples and that 705 and 741 OTUs were unique to the

CHB yellow tongue coating and white tongue coating patients, respectively (Figure 1C). Most of the tongue coating microbiota were assigned to the Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Fusobacteria phyla (relative abundance > 1% of the total DNA sequences, Figure 2A). In total, 112 genera were classified, and most of the genera were present in low abundance in the oral microbiota samples.

A Metastats analysis was used to detect differentially abundant features at different taxon levels. Compared with the white tongue patients, the relative abundance of Bacteroidetes was decreased in the CHB yellow tongue coating patients, whereas the abundance of Proteobacteria and Gracilibacteria were increased (Figure 2B). At the family level, Neisseriaceae and Gracilibacteria_bacterium_oral_taxon_873 were dominant in the CHB yellow tongue coating and had prevalent differences compared with the abundances in the healthy controls and CHB white tongue coating patients, whereas SR1_bacterium_canine_oral_taxon_380 was decreased. At the genus level, *Neisseria* and *Gracilibacteria_bacterium_oral_taxon_873* were enriched in the CHB yellow tongue coating samples compared with the healthy controls and the CHB white tongue coating patients. The relative abundance of *Actinomyces, Porphyromonas*, *Bergeyella*, *Centipeda, Alysiella, Bulleidia, Candidatus_Saccharibacteria_bacterium_UB2523* and *Pseudoramibacter* in the CHB white tongue coating samples were significantly different
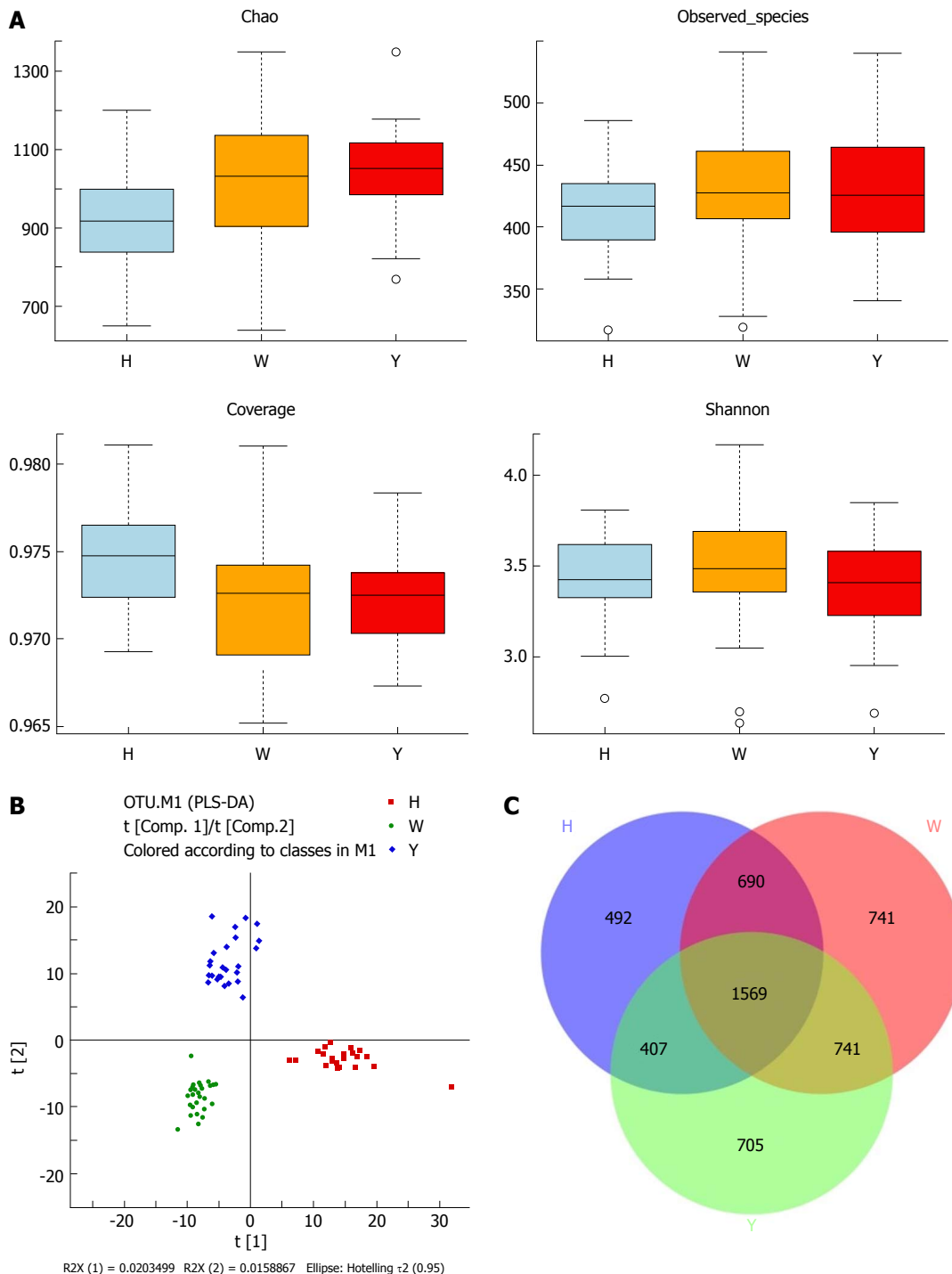
Figure 1 Overview of the tongue coating microbiota among groups. A: Detailed characteristics of alpha diversity; B: PLS-DA discriminant analysis plot; C: Venn diagram based on OTUs. PLS-DA: Partial least squares-discriminant analysis; OTUs: Operational taxonomic units.

from those of the healthy controls, but no differences were found compared to the abundances of the CHB yellow tongue coating patients (Figure 3A). We used LEfSe to identify the specific OTUs associated with each group. A cladogram representative of the structure of the tongue coating microbiotas in the CHB yellow tongue coating and CHB white tongue coating patients and the healthy controls is shown in Figure 3B. According to the LDA values ($\log_{10}^{LDA \ score} > 4$), the tongue coating bacteria with advantages in the CHB yellow tongue coating

were Betaproteobacteria, Neisseriales, Neisseriaceae, Proteobacteria and *Neisseria*, the CHB white tongue coating samples were dominated by Bacteroidia and Bacteroidales, and the healthy controls were dominated by Actinomycetaceae, Actinomycetales and *Actinomyces* (Figure 3C).

Predicted microbial function
The gene functions in the tongue coating microbiotas were predicted by PICRUSt, and the results were com-

A

B

Figure 2 Taxonomic classification of the tongue coating microbiota at the phylum level. A: Taxonomic classification of the tongue-coating microbiota at the phylum level; B: Metastats analysis of differences in the relative proportions difference at the phylum level. [a]$P < 0.05$, [b]$P < 0.01$.

pared between groups (Figure 4). Microbes with greater relative abundances in the healthy controls had functions that were most related to carbohydrate metabolism (fructose and mannose metabolism). The microbiotas in the white tongue coatings were more likely than those in the yellow tongue coatings and healthy controls to have functionality related to renetic information processing, including the level-2 functionality of replication and repair (DNA replication proteins, homologous recombination and mismatch repair) and transcription (transcription machinery, ribosome and translation factors). In contrast, the microbiotas in the yellow tongue coatings were probably related to amino acid metabolism (alanine aspartate and glutamate metabolism, arginine and proline metabolism, cysteine and methionine metabolism, lysine biosynthesis, phenylalanine tyrosine and tryptophan biosynthesis, phenylalanine metabolism, tyrosine metabolism and valine leucine and isoleucine biosynthesis), membrane transport (bacterial secretion system and secretion system) and cell motility (bacterial motility proteins).

Metabolites in the tongue coatings

The typical total ion chromatograms (TICs) of the samples run in positive ion modes are shown in supplementary Figure 2A. Because the PCA did not distinguish

the groups well, the supervised PLS-DA model was used for further analysis and resulted in good separation of the CHB patients and healthy volunteers, however, the CHB yellow tongue coating and the white tongue coating patients were not completely distinguished (Supplementary Figure 2B). A total of 21 endogenous compounds were obtained with a VIP > 1 and P(corr) > 0.4 in the PLS-DA model (Table 2). The presence of 6 compounds differed between the CHB patients and healthy subjects ($P < 0.05$), and these compounds were mainly involved in histidine metabolism and methylhistidine metabolism ($P < 0.05$, Supplementary Figure 3). The metabolites were not significantly different between the CHB yellow and white tongue coating patients, but the number of amino acid metabolites was greater in the CHB yellow tongue coating patients than in the white tongue coating patients.

Associations of the tongue coating microbiotas with metabolites and clinical indices

To better understand the relationships among the tongue coating microbiota, metabolites and clinical indicators, we performed a correlation analysis of the differential microbiota at the family level with the metabolites and clinical indicators and constructed the related network (|correlation r| > 0.3, $P < 0.05$). The abundance of
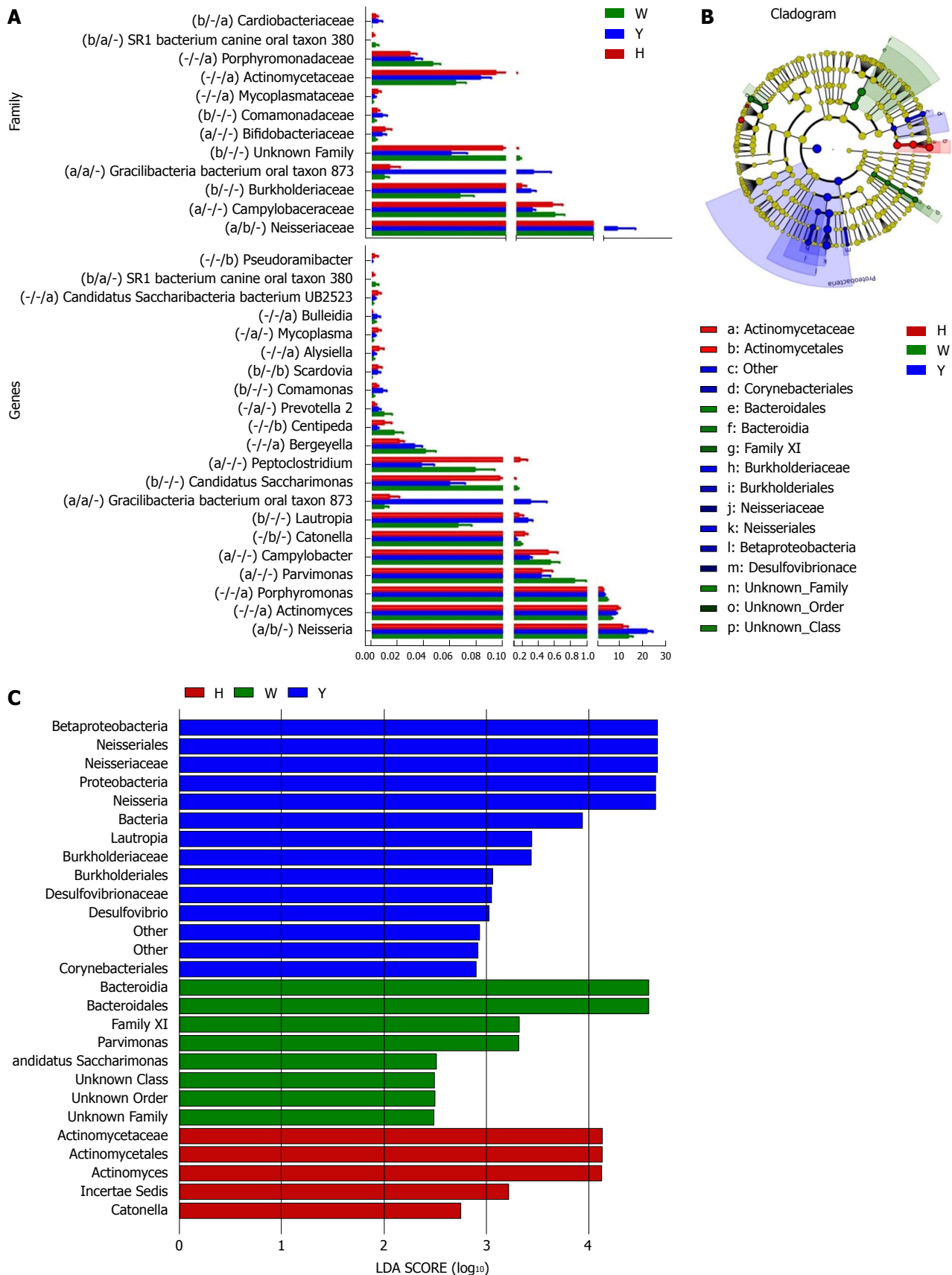
Figure 3 The most differentially abundant taxa in the chronic hepatitis B yellow and white tongue coating patients. A: Comparison of the relative abundance at the bacterial family and genus levels, the marks of significance from left to right in parentheses are the CHB yellow tongue coating patients *vs* the healthy controls, the CHB yellow tongue coating patients *vs* the CHB white tongue coating patients and the CHB white tongue coating patients *vs* the healthy controls. [a]*P* < 0.05, [b]*P* < 0.01, "−" indicates no significant difference based on the Metastats analysis; B: Taxonomic cladogram of the LEfSe analysis of the 16S sequences. (Red) healthy-control enriched taxa; (Green) CHB white tongue coating patients-enriched taxa; and (Blue) CHB yellow tongue coating patients-enriched taxa. C: LDA scores of enriched taxa in the healthy controls (Red), CHB white tongue coating patients (Green) and CHB yellow tongue coating patients (Blue). LDA: Linear discriminant analysis.

Figure 4 Linear discriminant analysis scores of the enriched microbial functions in the healthy-controls (Red), chronic hepatitis B white tongue coating patients (Green) and chronic hepatitis B yellow tongue coating patients (Blue). LDA: Linear discriminant analysis.

Table 2 Metabolites detected in the tongue coatings

Metabolites	Formula	Rt (min)	Type	KEGG compounds	VIP	P (corr)	CHB VS H		Healthy	CHB White tongue coating	CHB Yellow tongue coating
							P value	FC			
L-Proline	$C_5H_9NO_2$	83.3392	[M+H]+	C00148	1.04	-0.68	0.134	1.473	1.01 (0.13-12)	3 (0.16-20.14)	3.88 (0.09-15.28)
L-Valine	$C_5H_{11}NO_2$	120.7915	[M+H]+	C00183	1.07	-0.76	0.081	1.608	2.92 (0.82-17.97)	3.98 (1.13-38.65)	7.44 (1.08-31.3)
L-Isoleucine	$C_6H_{13}NO_2$	194.3545	[M+H]+	C00123	1.15	-0.81	0.069	1.641	5.55 (0.43-30.81)	7.23 (1.23-58.36)	13.28 (0.22-45.18)
L-Leucine	$C_6H_{13}NO_2$	212.576	[M+H]+	C00123	1.07	-0.81	0.029	1.715	8.9 (0.02-44.58)	14.65 (2.89-84.8)	21.6 (0.44-61.98)
Hypoxanthine	$C_5H_4N_4O$	154.305	[M+H]+	C00262	1.07	-0.80	0.039	2.218	0.42 (0.01-6.93)	1.31 (0.05-17.87)	2.46 (0.01-14.33)
Urocanic acid	$C_6H_6N_2O_2$	132.3225	[M+H]+	C00785	1.00	-0.78	0.044	2.587	0.62 (0.07-4.46)	0.93 (0.08-12.84)	1.93 (0.08-13.92)
L-Lysine	$C_6H_{14}N_2O_2$	64.124	[M+H]+	C00047	1.01	-0.72	0.683	1.510	0.08 (0.05-0.28)	0.08 (0.03-0.81)	0.08 (0.03-0.65)
Glutamate	$C_5H_9NO_4$	76.6581	[M+H]+	C00025	1.04	-0.75	0.159	1.955	0.14 (0.02-1.17)	0.2 (0-2.99)	0.39 (0-2.52)
Methionine	$C_5H_{11}NO_2S$	131.208	[M+H]+	C00073	1.16	-0.80	0.144	1.543	1.08 (0.01-7.21)	1.74 (0.13-10.21)	2.26 (0-9.75)
Xanthine	$C_5H_4N_4O_2$	183.102	[M+H]+	C00385	1.01	-0.73	0.151	2.058	0.09 (0-2.91)	0.24 (0-4.93)	0.47 (0-4.75)
L-Phenylalanine	$C_9H_{11}NO_2$	277.369	[M+H]+	C00079	1.17	-0.82	0.069	1.534	6.72 (0.83-24.15)	11.07 (1.84-51.35)	13.1 (0.31-36.18)
Glycyl-L-Valine	$C_7H_{14}N_2O_3$	205.364	[M+H]+		1.07	-0.70	0.078	1.548	0.07 (0-1.1)	0.15 (0-0.84)	0.19 (0-1.38)
Citrulline	$C_6H_{13}N_3O_3$	77.3246	[M+H]+	C00327	1.16	-0.74	0.216	1.486	0.12 (0-1.28)	0.14 (0.01-1.7)	0.3 (0-1.38)
L-Tyrosine	$C_9H_{11}NO_3$	204.441	[M+H]+	C00082	1.17	-0.81	0.095	1.512	2.63 (0.33-11.35)	4.36 (0.71-22.15)	5.67 (0.1-16.28)
Benzophenone	$C_{13}H_{10}O$	882.111	[M+H]+	C06354	1.19	0.81	< 0.001	0.752	0.25 (0.16-0.3)	0.19 (0.1-0.3)	0.18 (0.07-0.25)
L-Tryptophan	$C_{11}H_{12}N_2O_2$	338.856	[M+H]+	C00078	1.03	-0.74	0.23	1.940	0.57 (0.09-3.63)	0.74 (0.07-12.67)	1.11 (0.1-9.81)
12-Hydroxylauric acid	$C_{12}H_{24}O_3$	906.316	[M+H]+	C08317	1.12	0.72	< 0.001	0.697	10.81 (8.25-16.18)	7.3 (0.08-12.49)	7.61 (0.08-14.08)
N-Acetyl-D-glucosamine	$C_8H_{15}NO_6$	82.3978	[M+H]+	C03878	1.02	-0.39	0.936	0.942	0.04 (0-0.46)	0.07 (0-0.34)	0.07 (0-0.44)
L-cystathionine	$C_7H_{14}N_2O_4S$	836.2685	[M+H]+	C02291	1.10	0.67	0.24	0.919	0.48 (0.31-0.79)	0.48 (0.2-0.81)	0.43 (0.22-0.66)
S-adenosyl-L-methionine	$C_{15}H_{22}N_6O_5S$	66.1963	[M+H]+	C00019	1.02	0.79	0.029	0.888	9.98 (8.09-12.77)	9.2 (4.71-12.09)	8.58 (4.98-14.58)
Guanosine	$C_{10}H_{13}N_5O_5$	230.687	[M+H]-	C00387	1.01	0.02	0.824	0.763	3.02 (0.55-14.19)	2.98 (0-10.27)	3.52 (0-10.89)

Neisseriaceae, which was the dominant bacterial family in the CHB yellow tongue coating, was positively correlated with the serum HBV-DNA, TC, L-leucine, hypoxanthine, urocanic acid and methionine levels but negatively correlated with thebenzophenone and S-adenosyl-L-methionine levels. Another relatively abundant member of the microbiota in the CHB yellow tongue coating (Gracilibacteria_bacterium_oral_taxon_873) was positively correlated with the guanosine and BUN levels but negatively correlated with the S-adenosyl-L-methionine level (Figure 5).

DISCUSSION

Chinese medicine theory believes that tongue demonstration is a window of body change and that the oral microbiome is an integral part of the whole body's microbiome. Indeed, an increasing number of studies have found that many diseases, including atherosclerosis[15], rheumatoid arthritis[16], diabetes[17] and liver cirrhosis[18], are accompanied by disorder of the oral and intestinal microbiotas. Although the oral and gut communities share little taxonomic resemblance, the oral and intestinal microflora have general dynamic characteristics[19], the oral bacterial populations seed the gut[20], and the observed bacterial structures in the oral cavity or intestine can predict each other[21]. Because tongue diagnosis has the advantages of intuition and simplicity, some scholars have recently discovered the importance of the tongue image for the diagnosis and prognostic prediction of diseases[22,23]. The oral microbiota varies according to the different sampling sites with a high diversity[24], but as the tongue texture and tongue color form an important basis for diagnosis and treatment in TCM[25]. The treatments adopted for patients with white and yellow tongue coatings are different, thus, the tongue coating oral microbiotas were selected for analysis in our study, and the samples were grouped by the color of the tongue coating.

The complex and dynamic interactions between microbes and hosts are very important for maintaining homeostasis because microbiome dysbiosis may interact with the host's immune system and influence the degree of hepatic steatosis, inflammation and fibrosis[26]. Our recent study found that the gut microbiota was significantly altered in
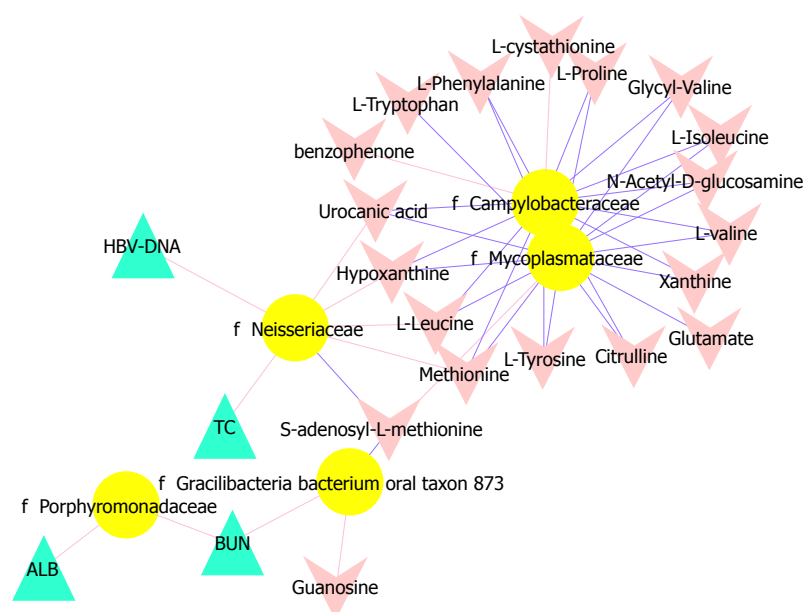
Figure 5 Associations of the tongue coating microbiotas with metabolites and clinical indices. Correlations were detected, and the indices with Spearman's correlation coefficients > 0.3 were visualized in Cytoscape. Triangles represent clinical indicators, arrows represent metabolites and dots represent oral microbial families. If the correlations are negative, the connecting line is blue; if the correlation is positive, the connecting line is red. The line thickness represents the size of the correlation coefficient.

CHB patients with low Child-Pugh scores[27]. Evidence is accumulating that the intestinal microbiome has good clinical prospects as a target for the treatment of chronic liver disease. Chinese scholars transplanted the intestinal flora of healthy volunteers into HBeAg-positive CHB patients with long-term antiviral treatment, and this approach showed a good effect on HBeAg seroclearance[28]. However, few studies have been performed on the oral microbiotas in CHB patients. In one of the limited reports, Ling *et al*[29] noted the decreased diversity of the oral microbiotas in supragingival plaque samples from patients with HBV-induced chronic liver disease. However, in this study, the richness and diversity of the tongue coating did not differ between the CHB patients and healthy controls. Ren *et al*[30] used 16S rRNA gene pyrosequencing and metagenomic sequencing to examine oral microbial compositions and their functional variations in children with halitosis. They found that the tongue coatings of the subjects with halitosis had greater bacterial richness than those of the healthy subjects, but the number of OTUs and the Chao levels were slightly lower in the saliva samples from the subjects with halitosis than in the healthy subjects. The microbiotas among individuals and habitats are highly personalized in the oral cavity[31]. Therefore, we concluded that the data differences in data might be due to the use of different sampling sites.

Taxonomic and predicted function differences were found between the CHB yellow and white tongue coating patients in the LEfSe analysis. The CHB yellow tongue coating patients had specific clinical and microbiota characteristics that were distinct from those of the white coating patients. First, the CHB patients with yellow tongue coatings had significantly higher viral titers but lower ALB levels than the white coating patients (*P* <

0.05). The remaining indicators of liver function, including TBIL, DBIL, ALT, AST, ALP, GGT and PT, exhibited elevated trend in the CHB yellow tongue coating patients. Because a yellow tongue is an important indicator for the diagnosis of CHB *damp heat* syndrome, the results are consistent with studies showing that the CHB *damp-heat* syndrome patients have higher ALT and AST levels[32], which indicats that these patients have greater liver damage. The correlation analysis between bacteria and metabolites also confirmed this outcome. The relatively dominant bacterial families in the CHB yellow tongue coating (Neisseriaceae and Gracilibacteria_bacterium _oral_taxon_873) were negatively correlated with the S-adenosyl-L-methionine level. S-adenosyl-L-methionine, which is also known as SAM or AdoMet, is a donor in the methylation reactions of all mammalian cells but is found most abundantly in the liver. Patients with chronic liver disease have reduced AdoMet levels, and reduced hepatic AdoMet levels can contribute to the development of oxidative stress, steatohepatitis, and hepatocellular carcinoma (HCC)[33]. In addition, according to TCM, a yellow tongue coating is a manifestation of TCM *hot* Syndrome, which has been reported to be involved in inflammation or an immune regulation imbalance in the host[34,35]. Jiang *et al*[3] compared the yellow and white tongue coating microbiomes with those of gastritis patients and found that yellow dense tongue coating bacteria were associated with TCM *hot* syndrome-related inflammation such as halitosis, tonsil infection and periodontal inflammation. Therefore, we therefore hypothesized that the patients with CHB yellow tongue coatings might have had more inflammation, although this speculation requires further confirmation. Second, the yellow tongue coating subjects showed similarities at the phylum level with a gut disorder in patients

with cirrhosis, which was manifested as a reduced abundance of Bacteroidetes and increased Proteobacteria content[18,26,36]. The LEfSe analysis suggested that Neisseriaceae was enriched in the yellow tongue coating patients and was positively correlated with the serum HBV-DNA level. Ling's research reported that members of Neisseriaceae were significantly more abundant in the oral microbiota of liver cirrhosis patients than in those of CHB patients[29]. Lv *et al*[37] found that Neisseriaceae, which are opportunistic pathogens, were enriched in primary biliary cirrhosis (PBC) patients. Third, according to the LDA score, the inferred metagenomic pathways that were enriched in the CHB yellow tongue coating patients were mainly those involved in amino acid metabolism, and the detected metabolites were mainly essential amino acids, including L-proline, L-valine, L-isoleucine, L-leucine, L-lysine, glutamate and L-tryptophan, and were generally present in larger amounts trend in the yellow tongue coatings. The relationship between amino acid metabolism and the CHB patients with yellow tongue coatings is unclear, and relevant literature reports are limited. However, one report noted that people with the TCM *damp-heat* syndrome people with HBV-related chronic liver diseases, whom mainly manifested yellow greasy tongue coatings, had increased leucine, phenylalanine and valine expression in urinary metabolomic studies[38], which indirectly explained the severe disordered amino acid metabolism in CHB patients with the yellow coating. Finally, this study found that membrane transport (secretion system) and cell motility (bacterial motility proteins) were enriched in the yellow coatings, which indicated that the CHB yellow coating patients might be more susceptible to bacterial colonization. The functional analysis of tongue coating flora and metabolites in CHB white tongue coating patients lacked correspondence, the underlying reason might be the microbiotas functionality in the CHB white tongue coating patients were most likely related to genetic information processing, which association with metabolites is relatively little.

Tongue diagnosis is a simple, non-invasive and valuable procedure that has been repeatedly verified by TCM clinical practitioners. We found that the yellow and white tongue coatings of CHB patients had microbiota compositional and functional differences in this study, but the sample sizes were small. In the future, we will expand the sample size to verify the results of this study. In addition, we will focus on the relationship between changes in the oral and intestinal microbiota in different tongue coating patients and explore the effects of oral microbiota variance on immunity and metabolic alterations in the body to further explain the modern theoretical mechanism of TCM tongue diagnosis. These studies will facilitate the development of therapeutic strategies for individualized treatment.

ARTICLE HIGHLIGHTS

Research background
Chronic hepatitis B is a major infectious disease in China and Chinese medicine

has a wide range of applications in the treatment of CHB. Tongue diagnosis has important guiding significance for clinical syndrome differentiation and drug use in TCM, but lacks scientific explanations. Some reports have found abnormalities in the microbiota or metabolites in pathological tongue coatings. However, integrated analyses of the pathological tongue coating microbiotas and metabolites have rarely been reported. Elucidating the tongue coatings micro-features differences will promote our understanding of the TCM tongue diagnosis and facilitate therapeutic strategies for individualized treatment.

Research motivation
The motivation of this study was to explore the microfeatures of different tongue coatings, which could promote our understanding of the TCM tongue diagnosis from a modern perspective.

Research objectives
The objective of this research was to elucidate tongue coating microbiota and metabolic differences in CHB patients with yellow or white tongue coatings.

Research methods
We collected tongue coating samples from 28 CHB yellow tongue coating patients and 25 CHB white tongue coating patients, and an additional 22 samples were collected from healthy controls. The tongue coating bacterial 16S ribosomal RNA gene V3 region was amplified and sequenced with the Ion Torrent PGM™ sequencing platform. The metabolites in the tongue coatings were examined using a LC-MS platform. The microbiota results were analyzed using Metastats analysis, the Kruskal-Wallis test and LEfSe analysis. The functionality of the microbiota was assessed using PiCRUST and compared among groups. The metabolomics enrichment analysis was performed with MetaboAnalyst 3.0 (http://www.metaboanalyst.ca). We correlated the microbiota with the clinical indices and metabolites and visualized the results with Spearman's correlation coefficients > 0.3 and *P* < 0.05 in Cytoscape.

Research results
This study found taxonomic and predicted function differences between the CHB yellow and white tongue coating patients. Distinct from those of the white coating patients, the CHB yellow tongue coating patients had specific clinical and microbiota characteristics. The microbiota of the CHB patients with yellow tongue coatings had similarities with a gut disorder in patients with cirrhosis at the phylum level, which manifested as a reduced abundance of Bacteroidetes and increased Proteobacteria content. Neisseriaceae, which is a dominant bacterial family enriched in yellow tongue coating patients, was positively correlated with the serum HBV-DNA level. The inferred metagenomic pathways enriched in the CHB yellow tongue coating patients were mainly those involved in amino acid metabolism, whereas the detected metabolites were mainly essential amino acids and generally were present in larger amounts than in the white tongue coatings.

Research conclusions
We found that the yellow and white tongue coatings of CHB patients had microbiota compositional and functional differences in this study.

Research perspectives
The sample size in this study was small. In the future, we will expand the sample size to further verify the results and focus on the relationship between changes in the oral and intestinal microbiotas to explore the effects of oral microbiota variance on immunity and metabolic alterations in the body. These studies will further explain the modern theoretical mechanism of TCM tongue diagnosis.

REFERENCES

1　**Ye J**, Cai X, Cao P. Problems and prospects of current studies on the microecology of tongue coating. *Chin Med* 2014; **9**: 9 [PMID: 24597827 DOI: 10.1186/1749-8546-9-9]

2　**Lira-Junior R**, Boström EA. Oral-gut connection: one step closer to an integrated view of the gastrointestinal tract? *Mucosal Immunol* 2018; **11**: 316-318 [PMID: 29297500 DOI: 10.1038/mi.2017.116]

3　**Jiang B**, Liang X, Chen Y, Ma T, Liu L, Li J, Jiang R, Chen T, Zhang X, Li S. Integrating next-generation sequencing and traditional tongue diagnosis to determine tongue coating microbiome. *Sci Rep* 2012; **2**: 936 [PMID: 23226834 DOI: 10.1038/srep00936]

4　**Sun ZM**, Zhao J, Qian P, Wang YQ, Zhang WF, Guo CR, Pang XY, Wang SC, Li FF, Li Q. Metabolic markers and microecological characteristics of tongue coating in patients with chronic gastritis. *BMC Complement Altern Med* 2013; **13**: 227 [PMID: 24041039 DOI: 10.1186/1472-6882-13-227]

5　**Wang FS**, Fan JG, Zhang Z, Gao B, Wang HY. The global burden of liver disease: the major impact of China. *Hepatology* 2014; **60**: 2099-2108 [PMID: 25164003 DOI: 10.1002/hep.27406]

6　**Chassaing B**, Etienne-Mesmin L, Gewirtz AT. Microbiota-liver axis in hepatic disease. *Hepatology* 2014; **59**: 328-339 [PMID: 23703735 DOI: 10.1002/hep.26494]

7　**Bajaj JS**, Betrapally NS, Hylemon PB, Heuman DM, Daita K, White MB, Unser A, Thacker LR, Sanyal AJ, Kang DJ, Sikaroodi M, Gillevet PM. Salivary microbiota reflects changes in gut microbiota in cirrhosis with hepatic encephalopathy. *Hepatology* 2015; **62**: 1260-1271 [PMID: 25820757 DOI: 10.1002/hep.27819]

8　**Tsai TY**, Livneh H, Hung TH, Lin IH, Lu MC, Yeh CC. Associations between prescribed Chinese herbal medicine and risk of hepatocellular carcinoma in patients with chronic hepatitis B: a nationwide population-based cohort study. *BMJ Open* 2017; **7**: e014571 [PMID: 28122837 DOI: 10.1136/bmjopen-2016-014571]

9　**Kang H**, Zhao Y, Li C, Chen Y, Tang K, Yang L, Ma C, Peng J, Zhu R, Liu Q, Hu Y, Cao Z. Integrating clinical indexes into four-diagnostic information contributes to the Traditional Chinese Medicine (TCM) syndrome diagnosis of chronic hepatitis B. *Sci Rep* 2015; **5**: 9395 [PMID: 25797918 DOI: 10.1038/srep09395]

10　**Zhao Y**, Kang H, Peng JH, Xu L, Cao ZW, Hu YY. Key symptoms selection for two major syndromes diagnosis of Chinese medicine in chronic hepatitis B. *Chin J Integr Med* 2017; **23**: 253-260 [PMID: 27225292 DOI: 10.1007/s11655-016-2253-3]

11　**Amato KR**, Yeoman CJ, Kent A, Righini N, Carbonero F, Estrada A, Gaskins HR, Stumpf RM, Yildirim S, Torralba M, Gillis M, Wilson BA, Nelson KE, White BA, Leigh SR. Habitat degradation impacts black howler monkey (Alouatta pigra) gastrointestinal microbiomes. *ISME J* 2013; **7**: 1344-1353 [PMID: 23486247 DOI: 10.1038/ismej.2013.16]

12　**Segata N**, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. Metagenomic biomarker discovery and explanation. *Genome Biol* 2011; **12**: R60 [PMID: 21702898 DOI: 10.1186/gb-2011-12-6-r60]

13　**Langille MG**, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Vega Thurber RL, Knight R, Beiko RG, Huttenhower C. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat Biotechnol* 2013; **31**: 814-821 [PMID: 23975157 DOI: 10.1038/nbt.2676]

14　**Xia J**, Sinelnikov IV, Han B, Wishart DS. MetaboAnalyst 3.0--making metabolomics more meaningful. *Nucleic Acids Res* 2015; **43**: W251-W257 [PMID: 25897128 DOI: 10.1093/nar/gkv380]

15　**Koren O**, Spor A, Felin J, Fåk F, Stombaugh J, Tremaroli V, Behre CJ, Knight R, Fagerberg B, Ley RE, Bäckhed F. Human oral, gut, and plaque microbiota in patients with atherosclerosis. *Proc Natl Acad Sci U S A* 2011; **108 Suppl 1**: 4592-4598 [PMID: 20937873 DOI: 10.1073/pnas.1011383107]

16　**Zhang X**, Zhang D, Jia H, Feng Q, Wang D, Liang D, Wu X, Li J, Tang L, Li Y, Lan Z, Chen B, Li Y, Zhong H, Xie H, Jie Z, Chen W, Tang S, Xu X, Wang X, Cai X, Liu S, Xia Y, Li J, Qiao X, Al-Aama JY, Chen H, Wang L, Wu QJ, Zhang F, Zheng W, Li Y, Zhang M,

Luo G, Xue W, Xiao L, Li J, Chen W, Xu X, Yin Y, Yang H, Wang J, Kristiansen K, Liu L, Li T, Huang Q, Li Y, Wang J. The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. *Nat Med* 2015; **21**: 895-905 [PMID: 26214836 DOI: 10.1038/nm.3914]

17　**Xiao E**, Mattos M, Vieira GHA, Chen S, Corrêa JD, Wu Y, Albiero ML, Bittinger K, Graves DT. Diabetes Enhances IL-17 Expression and Alters the Oral Microbiome to Increase Its Pathogenicity. *Cell Host Microbe* 2017; **22**: 120-128.e4 [PMID: 28704648 DOI: 10.1016/j.chom.2017.06.014]

18　**Qin N**, Yang F, Li A, Prifti E, Chen Y, Shao L, Guo J, Le Chatelier E, Yao J, Wu L, Zhou J, Ni S, Liu L, Pons N, Batto JM, Kennedy SP, Leonard P, Yuan C, Ding W, Chen Y, Hu X, Zheng B, Qian G, Xu W, Ehrlich SD, Zheng S, Li L. Alterations of the human gut microbiome in liver cirrhosis. *Nature* 2014; **513**: 59-64 [PMID: 25079328 DOI: 10.1038/nature13568]

19　**Bashan A**, Gibson TE, Friedman J, Carey VJ, Weiss ST, Hohmann EL, Liu YY. Universality of human microbial dynamics. *Nature* 2016; **534**: 259-262 [PMID: 27279224 DOI: 10.1038/nature18301]

20　**Atarashi K**, Suda W, Luo C, Kawaguchi T, Motoo I, Narushima S, Kiguchi Y, Yasuma K, Watanabe E, Tanoue T, Thaiss CA, Sato M, Toyooka K, Said HS, Yamagami H, Rice SA, Gevers D, Johnson RC, Segre JA, Chen K, Kolls JK, Elinav E, Morita H, Xavier RJ, Hattori M, Honda K. Ectopic colonization of oral bacteria in the intestine drives TH1 cell induction and inflammation. *Science* 2017; **358**: 359-365 [PMID: 29051379 DOI: 10.1126/science.aan4526]

21　**Ding T**, Schloss PD. Dynamics and associations of microbial community types across the human body. *Nature* 2014; **509**: 357-360 [PMID: 24739969 DOI: 10.1038/nature13178]

22　**Han S**, Yang X, Qi Q, Pan Y, Chen Y, Shen J, Liao H, Ji Z. Potential screening and early diagnosis method for cancer: Tongue diagnosis. *Int J Oncol* 2016; **48**: 2257-2264 [PMID: 27035407 DOI: 10.3892/ijo.2016.3466]

23　**RiYang L**, HangYing Y, JunYan Q, YaYu L, YuHui W, YaZhen Y, JiaZhen Y, Jin Y, Jun N, DongRong Y. Association between tongue coating thickness and clinical characteristics among idiopathic membranous nephropathy patients. *J Ethnopharmacol* 2015; **171**: 125-130 [PMID: 25997785 DOI: 10.1016/j.jep.2015.05.008]

24　**Human Microbiome Project Consortium**. Structure, function and diversity of the healthy human microbiome. *Nature* 2012; **486**: 207-214 [PMID: 22699609 DOI: 10.1038/nature11234]

25　**Lee TC**, Lo LC, Wu FC. Traditional Chinese Medicine for Metabolic Syndrome via TCM Pattern Differentiation: Tongue Diagnosis for Predictor. *Evid Based Complement Alternat Med* 2016; **2016**: 1971295 [PMID: 27313640 DOI: 10.1155/2016/1971295]

26　**Tilg H**, Cani PD, Mayer EA. Gut microbiome and liver diseases. *Gut* 2016; **65**: 2035-2044 [PMID: 27802157 DOI: 10.1136/gutjnl-2016-312729]

27　**Wang J**, Wang Y, Zhang X, Liu J, Zhang Q, Zhao Y, Peng J, Feng Q, Dai J, Sun S, Zhao Y, Zhao L, Zhang Y, Hu Y, Zhang M. Gut Microbial Dysbiosis Is Associated with Altered Hepatic Functions and Serum Metabolites in Chronic Hepatitis B Patients. *Front Microbiol* 2017; **8**: 2222 [PMID: 29180991 DOI: 10.3389/fmicb.2017.02222]

28　**Ren YD**, Ye ZS, Yang LZ, Jin LX, Wei WJ, Deng YY, Chen XX, Xiao CX, Yu XF, Xu HZ, Xu LZ, Tang YN, Zhou F, Wang XL, Chen MY, Chen LG, Hong MZ, Ren JL, Pan JS. Fecal microbiota transplantation induces hepatitis B virus e-antigen (HBeAg) clearance in patients with positive HBeAg after long-term antiviral therapy. *Hepatology* 2017; **65**: 1765-1768 [PMID: 28027582 DOI: 10.1002/hep.29008]

29　**Ling Z**, Liu X, Cheng Y, Jiang X, Jiang H, Wang Y, Li L. Decreased Diversity of the Oral Microbiota of Patients with Hepatitis B Virus-Induced Chronic Liver Disease: A Pilot Project. *Sci Rep* 2015; **5**: 17098 [PMID: 26606973 DOI: 10.1038/srep17098]

30　**Ren W**, Xun Z, Wang Z, Zhang Q, Liu X, Zheng H, Zhang Q, Zhang Y, Zhang L, Wu C, Zheng S, Qin N, Ehrlich SD, Li Y, He X, Xu T, Chen T, Chen F. Tongue Coating and the Salivary Microbial Communities Vary in Children with Halitosis. *Sci Rep* 2016; **6**: 24481 [PMID: 27080513 DOI: 10.1038/srep24481]

31   **Aas JA**, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol* 2005; **43**: 5721-5732 [PMID: 16272510 DOI: 10.1128/JCM.43.11.5721-5732. 2005]

32   **Sun YG**, Sun YL. Progress of the study on the relationship between TCM Syndromes of chronic hepatitis B and objective indicators. *Zhongyiyao Daobao* 2011; **17**: 76-78 [DOI: 10.13862/j.cnki. cn43-1446/r.2011.12.027]

33   **Lu SC**, Mato JM. S-adenosylmethionine in liver health, injury, and cancer. *Physiol Rev* 2012; **92**: 1515-1542 [PMID: 23073625 DOI: 10.1152/physrev.00047.2011]

34   **Li R**, Ma T, Gu J, Liang X, Li S. Imbalanced network biomarkers for traditional Chinese medicine Syndrome in gastritis patients. *Sci Rep* 2013; **3**: 1543 [PMID: 23529020 DOI: 10.1038/srep01543]

35   **Lu YY**, Zhao Y, Song YN, Dong S, Wei B, Chen QL, Hu YY, Su SB. Serum cytokine profiling analysis for zheng differentiation in chronic hepatitis B. *Chin Med* 2015; **10**: 24 [PMID: 26312063 DOI: 10.1186/s13020-015-0055-8]

36   **Chen Y**, Yang F, Lu H, Wang B, Chen Y, Lei D, Wang Y, Zhu B, Li L. Characterization of fecal microbial communities in patients with liver cirrhosis. *Hepatology* 2011; **54**: 562-572 [PMID: 21574172 DOI: 10.1002/hep.24423]

37   **Lv LX**, Fang DQ, Shi D, Chen DY, Yan R, Zhu YX, Chen YF, Shao L, Guo FF, Wu WR, Li A, Shi HY, Jiang XW, Jiang HY, Xiao YH, Zheng SS, Li LJ. Alterations and correlations of the gut microbiome, metabolism and immunity in patients with primary biliary cirrhosis. *Environ Microbiol* 2016; **18**: 2272-2286 [PMID: 27243236 DOI: 10.1111/1462-2920.13401]

38   **Guo Z**, Wang WY, Dai JY, Fan ZQ, Cao HJ, Sun SJ, Zhang YY, Xu LM, Hu YY, Su SB. Investigation on Urinary Metabolomic in Syndrome Types of Cirrhosis Caused by Hepatitis B. *Shijie Kexue Jishu-Zhongyiyao Xiandaihua* 2012; **14**: 1282-1287

CASE REPORT

Large heterotopic gastric mucosa and a concomitant diverticulum in the rectum: Clinical experience and endoscopic management

Wen-Guo Chen, Hua-Tuo Zhu, Ming Yang, Guo-Qiang Xu, Li-Hua Chen, Hong-Tan Chen

Wen-Guo Chen, Hua-Tuo Zhu, Ming Yang, Guo-Qiang Xu, Li-Hua Chen, Hong-Tan Chen, Department of Gastroenterology, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

ORCID number: Wen-Guo Chen (0000-0001-6286-1808); Hua-Tuo Zhu (0000-0002-5098-7734); Ming Yang (0000-0001-6417-9669); Guo-Qiang Xu (0000-0002-7553-415X); Li-Hua Chen (0000-0003-0498-4361); Hong-Tan Chen (0000-0002-0633-2343).

Author contributions: Chen WG wrote the paper; Zhu HT collected and analyzed the data; Yang M performed the research; Xu GQ and Chen LH were study supervisors; Chen HT conceived and designed the study; all the authors approved the final version of the article to be published.

Informed consent statement: Written informed consent was provided by the patient prior to study inclusion. All details that might disclose the identity of the subject were omitted or anonymized.

Conflict-of-interest statement: The authors of this manuscript have no conflicts of interest to disclose.

CARE Checklist (2013) statement: The authors have read the CARE Checklist (2013), and the manuscript was prepared and revised according to the CARE Checklist (2013).

Manuscript source: Unsolicited manuscript

Correspondence to: Hong-Tan Chen, MD, Chief Doctor, Department of Gastroenterology, The First Affiliated Hospital, College of Medicine, Zhejiang University, Qingchun road, Hangzhou 310003, Zhejiang Province, China. 1510026@zju.edu.cn.
Telephone: +86-571-87236729
Fax: +86-571-87236611

Abstract

Heterotopic gastric mucosa (HGM) in the rectum is an extremely rare clinical entity which may be missed or misdiagnosed due to a lack of knowledge. In the present study, a 14-year-old girl visited our hospital due to a 5-year history of repeated hematochezia. Colonoscopy showed a solitary superficial depressed lesion approximately 5 cm in size and a concomitant 1.5 cm deep diverticulum in the rectum. Histological examination of the endoscopic biopsy showed typical ectopic gastric mucosa in the depressed lesion and inside the diverticulum. Narrow band imaging further confirmed the histological results. Endoscopic ultrasound indicated that the lesion originated from the mucosal layer, and partially involved the submucosal layer. Endoscopic submucosal dissection was performed in this patient due to the large size and shape of the lesion. No bleeding, perforation or other adverse events were observed. The presence of HGM in the diverticular cavity greatly increased the surgical difficulty. A literature review was also carried out in our study.

Key words: Endoscopic submucosal dissection; Rectum;

Helicobacter pylori; Endoscopy; Heterotopic gastric mucosa

Core tip: Heterotopic gastric mucosa (HGM) in the rectum is an extremely rare clinical entity with unclear pathogenesis and no standard guidelines regarding optimal treatment. We present a patient with HGM and a concomitant diverticulum in the rectum, indicated its endoscopic ultrasound and narrow band imaging characteristics. Endoscopic submucosal dissection (ESD) was performed in this patient due to the large size and shape of the lesion. This is the first report of the resection of HGM and a concomitant diverticulum by ESD. A literature review was also carried out to investigate the clinical characteristics, diagnosis and management of HGM in the rectum.

INTRODUCTION

Heterotopic gastric mucosa (HGM) is the most commonly reported epithelial heterotopia and has been described as heteroplasia in organogenesis or metaplasia during the repair of damaged epithelium[1]. Although HGM may be congenital or acquired, the true pathogenesis of this condition remains unclear. HGM has been reported to be ubiquitous throughout the gastrointestinal (GI) tract from the oral cavity to the anorectum and can also involve the pancreas, biliary tract, gallbladder, scrotum, and mediastinum[2]. In the GI tract, HGM is predominantly observed in the esophagus, duodenum, Meckel's diverticulum, and in gastrointestinal duplication[3]. HGM in the rectum is an extremely rare clinical entity, which may be missed or misdiagnosed due to a lack of knowledge. To the best of our knowledge, only 50-100 cases of HGM in the rectum have been reported in the literature since the first description by Ewell and Jackson in 1939[4]. The majority of these patients were from Europe and North America.

In this report, we present the case of a young girl with a large 5 cm HGM in the rectum. Interestingly, a diverticular cavity 1.5 cm deep was found which was also lined with HGM. We report our clinical experience of this patient followed by a review of the recent literature on this entity. The clinical characteristics, endoscopic findings, diagnosis, treatment, and the relationship with Helicobacter pylori (*H. pylori*) infection and neoplastic transformation were analyzed to enhance physician awareness. In addition, our therapeutic approach using endoscopic submucosal dissection (ESD) which, to our knowledge, has been rarely reported.

CASE REPORT

A 14-year-old girl visited our hospital due to a 5-year history of repeated hematochezia. The bleeding episodes occurred intermittently without abdominal pain, mucous, pus or other symptoms. Her medical and family history was unremarkable. Carbohydrate antigen (CA) 19-9 was 61.2 U/mL (normal range 0-37 U/mL), hemoglobin was 116 g/L (normal range 113-151 g/L) and stool occult blood examination was weakly positive. Other laboratory examinations were normal. Abdominal computed tomography (CT), gastroscopy, and capsule endoscopy (CE) revealed no obvious GI lesions. A technetium-99m (Tc-99m) pertechnetate scan for Meckel's diverticulum and other sites of ectopic gastric mucosa was normal. On rectal examination, the patient had no anal fissures, hemorrhoids or fistulae.

At colonoscopy, a solitary superficial depressed lesion, approximately 5 cm in size, with a diverticular cavity was identified in the posterior wall of the lower part of the rectum proximal to the dentate line of the anal canal. The lesion was well-circumscribed, covering 1/3 of the circumference of the enteric cavity. Mucosa in the lesion appeared hyperemia and erythematous, with nodule changes. The tissue in the border of the lesion was slightly raised. The diverticulum was located in the anal side of the lesion, and was 1.5 cm deep and 2 cm in diameter. The mucosa was similar to the surrounding tissue. Histological examination of the endoscopic biopsy showed typical ectopic gastric mucosa in the depressed lesion and inside the diverticulum. Intestinal metaplasia, dysplasia, malignancy and colonization by *H. pylori* were not observed. Thus, the diagnosis of this rare entity was made on the basis of histological findings. Narrow band imaging (NBI) examination further confirmed the histological results. By means of NBI-magnifying endoscopy examination, it could visualize the microsurface and microvascular architecture immediately.

In this case, the NBI result showed the major gastric fundic-type mucosa (honey comb microvascular, small round microsurface) and focal pyloric-type mucosa (coiled microvascular, polygon microsurface) which lined the rectal mucosa. Endoscopic ultrasound (EUS) showed that the lesion originated from the mucosal layer, and partially involved the submucosal layer. Thickening of the mucosal layer with hypoechoic changes was observed in the intestinal wall of the lesion.

Although treatment with proton pump inhibitors or H2 receptor blockers may ameliorate the symptoms, we decided to remove the lesion by endoscopic resection due to repeated bleeding. As a result of its endoscopic features, ESD was performed in order to ensure complete resection. Unlike the routine ESD procedure used in a lateral spreading tumor (LST), the presence of the diver-
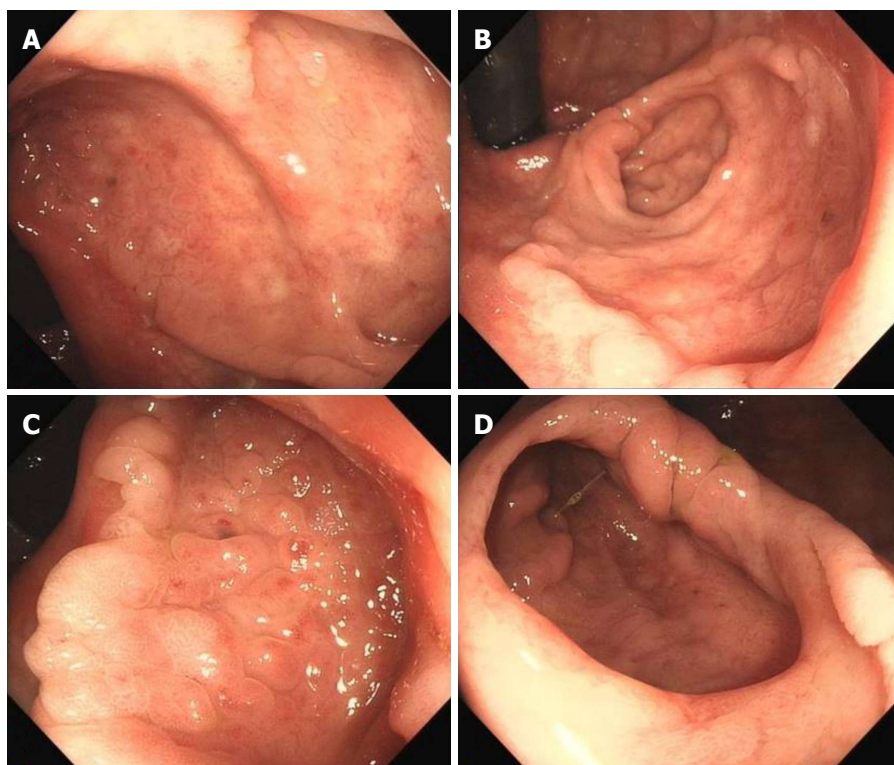
Figure 1 Endoscopic imaging. A and B: The lesion was observed by colonoscopy in the normal/reverse position; C: The slightly raised border, hyperemia and erythematous, nodule changed mucosa in the depressed lesion; D: Diverticulum in the heterotopic gastric mucosa lesion.

ticulum greatly increased the technical difficulties. After the lesion was marked with a dual knife, a mixture of glycerol fructose solution, indigo carmine and epinephrine were injected into the submucosal layer. The lifting sign was positive in this lesion which indicated that the muscularis propria was not involved. A circumferential incision was then performed. The submucosal dissection was started from the peripheral margin to the base of the diverticulum. When the dissection reached the anal side of the lesion near the anal canal, lidocaine was injected into the submucosal layer to relieve anal pain. Due to the narrow space in the diverticular cavity, ESD could not be accomplished. At the base of the diverticulum, piecemeal resection with snare was performed. In order to reduce residual HGM in the diverticulum, argon plasma coagulation (APC) was carried out in the interior of the diverticulum. Due to the hypervascular characteristics of the rectum, the full extent of the wound was carefully inspected, and a hemostatic clamp was used to manage the remnants of vessels. Finally, the lesion was resected without bleeding, perforation or other adverse events. Histological evaluation of the resected specimen revealed ectopic gastric mucosa of the fundic-type.

This study was approved by the Ethics Committee and Institutional Review Board of the First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China. Informed consent was obtained from the patient, and the record/information of the patient was anonymized and de-identified prior to analysis (Figures 1-5).

DISCUSSION

HGM has been reported to occur throughout the alimentary tract, but is mainly detected in the upper esophageal sphincter and is easily ignored by endoscopists if the gastroscope is withdrawn quickly without care. The incidence of HGM in the esophagus varies widely from 0.1% to 13.8% and HGM in the duodenum varies from 0.5% to 8.9% in published series[5]. HGM of the large bowel is infrequent, and is mostly reported in the rectum. Due to the limited number of patients, there is a lack of epidemiological studies and published data on HGM of the rectum. In a review of published reports, males were more commonly affected, and patients were predominantly children and adolescents[6,7]. Several hypotheses have been proposed to explain the origin of HGM, and false differentiation of pluripotent primitive endoderm stem cells is the most plausible and accepted hypothesis. Errors during embryonic development or metaplasia of rectal mucosa following mucosal injury are other mechanisms for the development of HGM[8].

The clinical presentation of HGM varies and depends on lesion size and location, and most are secondary to hydrochloric acid secretion. More than 90% of reported cases in the rectum were symptomatic[9]. The most common symptom was rectal bleeding, which also occurred in our case. Bleeding is usually painless, slight, intermittent and of varying duration. Other common symptoms include abdominal pain, diarrhea, tenesmus, perianal ulceration, anal pruritus, fistula, and bowel
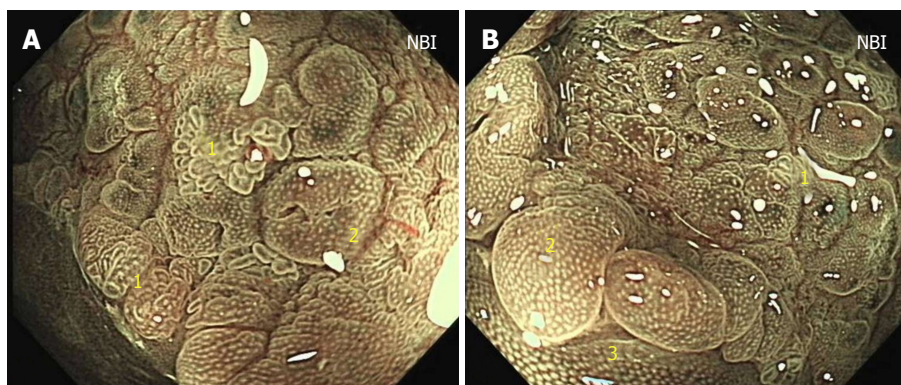
Figure 2 Narrow band imaging examinations. NBI showed that the lesion mainly consisted of gastric fundic-type mucosa with focal pyloric-type mucosa (1: Pyloric-type mucosa; 2: Fundic-type mucosa; 3: Rectal mucosa). NBI: Narrow band imaging.
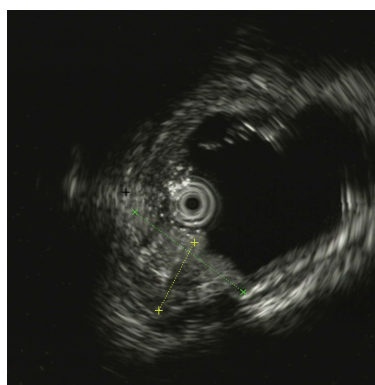

Figure 3 Endoscopic ultrasound examinations. Endoscopic ultrasound indicated that the lesion originated from the mucosal layer and partially involved the submucosal layer.

perforation. The report by Federico Iacopini showed that patients with specific anorectal symptoms were significantly younger than asymptomatic patients or patients with nonspecific abdominal symptoms and were frequently pediatrics (≤ 18 years)[10].

Endoscopically, HGM usually appears as an inlet patch in the proximal esophagus, a nodular mass in the duodenum, or a line in the interior of Meckel's diverticulum[11]. In the rectum, HGM lesions may be identified as ulcers, polyps, diverticula, and flat or depression lesions with a reddish-appearance. HGM mimicking a rectal submucosal tumor has also been reported[12]. Our patient, who had a 5 cm superficial depressed lesion and a concomitant 1.5 cm deep diverticulum, is the first reported case with this condition. The lesion was well-circumscribed with a slightly raised border and hyperemia, erythematous, and nodule altered mucosa. In most reported cases, the HGM lesion in the large intestine was solitary. Murray described a girl with repeated episodes of rectal bleeding due to HGM in the rectum, sigmoid colon, ascending colon, and cecum[13]. HGM is generally localized in the right posterior wall of the rectum and more than 5 cm above the anal verge[14]. EUS can provide valuable information for the diagnosis of HGM and can indicate the appropriate management.

In general, most lesions are found within the mucosal layer, and may involve the submucosal layer. EUS images have indicated a hypoechoic pattern change, and several small anechoic areas may be detected due to dilated gastric-type glands, which are characteristic of HGM[15]. Thickening of the mucosal layer can also be seen in EUS images. NBI and NBI-magnifying endoscopy are useful tools for the diagnosis of HGM and can differentiate gastric-type glands from the intestinal mucosa. To our knowledge, this case is also the first report of the use of NBI in HGM of the rectum. NBI clearly showed gastric fundic-type and pyloric-type mucosa lining the rectum and helped us to make a definitive diagnosis.

For the diagnosis of HGM in the rectum, histopathological examination is the gold standard and provides fragments of rectal mucosa coexisting with gastric mucosa. In a literature report, fundic-type mucosa was the most common histologic type of HGM, and was characterized by oxyntic features other than antral- or transitional-type[16]. Histopathology in our case also mainly showed fundic-type mucosa. Tc-99m pertechnetate abdominal scintigraphy is important in the diagnosis and localization of ectopic gastric mucosa, especially in Meckel's diverticulum. In the study by Sciarra *et al*[17], the sensitivity of Tc-99m scintigraphy ranged from 50% to 91%, which may have depended on the location and size of the heterotopic tissue. Our patient had a negative result with no increased uptake of foci in the GI tract. In addition to NBI, confocal laser endomicroscopy (CLE) is a new endoscopic tool that allows magnification of 1000 times with the aid of a miniaturized confocal microscope inserted into the biopsy canal of a conventional endoscope. Through high-resolution and high-magnification assessment, it can provide images that can reliably predict histology without the need for biopsies[18]. Thus, HGM in the rectum may be correctly detected using CLE.

No current guidelines regarding the optimal treatment of HGM in the rectum are available due to reported sporadic cases. By the inhibition of gastric acid secretion, H2 receptor blockers or proton pump inhibitors may ameliorate or eliminate symptoms caused by HGM in
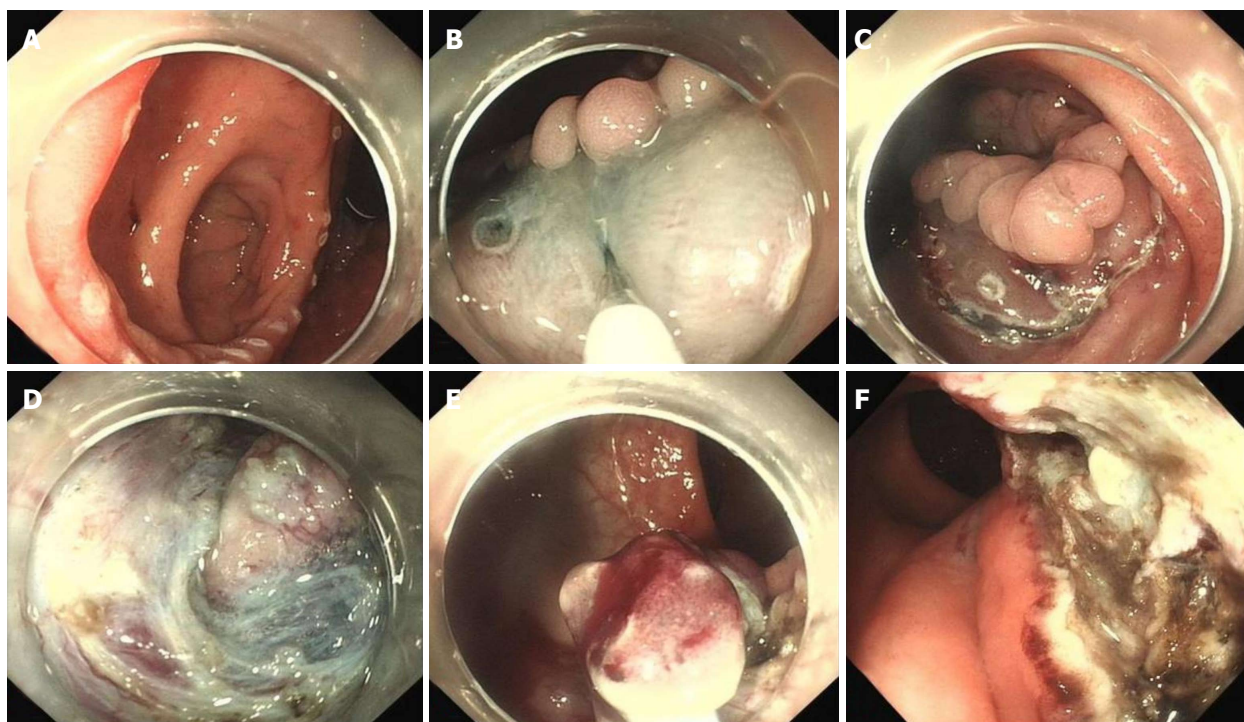
Figure 4 Endoscopic submucosal dissection of heterotopic gastric mucosa. A: Lesion marker; B: Submucosal layer injection; C: Circumferential incision; D: Dissection in the diverticular cavity; E: Piecemeal resection with a snare; F: Wound management.
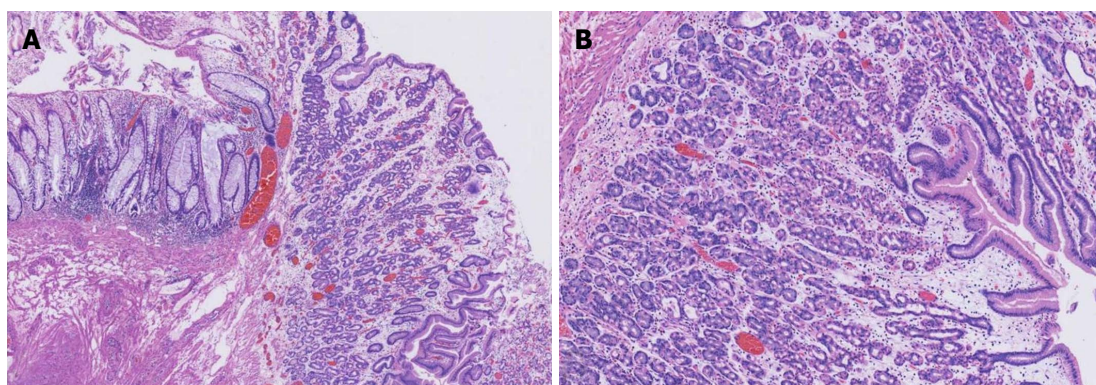

Figure 5 Histological examinations. Histologically, the specimen resected by endoscopic submucosal dissection showed ectopic gastric mucosa of the fundic-type.

the rectum. In most reported cases, rectal bleeding was successfully treated with proton pump inhibitor therapy and ulcer healing was achieved. However, recurrence can develop following the cessation of therapy and medication has failed to resolve heterotopic mucosa. In the surveillance report by Sauer *et al*[19], macroscopically the size of the lesion was unchanged after 4 mo of acid inhibition. Therefore, medical therapy relieves symptoms only in the short-term and could be used, to some extent, before definitive HGM excision. Endoscopic resection or surgical excision is generally considered the treatment of choice and has resulted in complete relief following removal of the HGM. Conventional endoscopic interventions such as endoscopic snare resection (polypectomy or endoscopic mucosal resection[20]) and ablation with APC are indicated in HGM patients mimicking polyps and small superficial lesions. ESD, enables *en*

bloc resection of superficial lesions especially in early GI cancer and LST, and could also be used in the treatment of HGM. To date, HGM in the rectum treated with ESD has been reported in only 2 cases[10,12]. Surgery is another definitive treatment and can achieve complete resection of the lesion. However, given the concerns related to this benign entity, excessive surgery should be viewed with great caution, and would be the preferred approach in HGM with rectal duplication or serious complications such as major gastrointestinal bleeding, perforation, abscess or fistula, and for lesions following failed endoscopic resection.

In the present study, ESD was performed in our patient due to the large size and shape of the lesion. The presence of HGM in the diverticular cavity greatly increased the surgical difficulty. In addition, gastric acid secreted by HGM can induce chronic gastritis and

ulcerative complications that may lead to submucosal fibrosis and further influence the submucosal dissection. Lesion marker and submucosal layer injection were performed used a dual knife as in conventional ESD. We attempted dissection from the oral side and anal side separately to the base of the diverticulum to the best of our ability. Based on our previous experience, lidocaine was injected into the submucosal layer in order to relieve anal pain during dissection of the rectum near the anal canal due to its rich nerve supply. *En bloc* resection could not be completed due to the narrow space in the diverticulum, and piecemeal resection with a snare was feasible. Finally, endoscopic ablation of the lesion with low wattage APC was performed to manage possible residual HGM in the interior of the diverticulum. The lesion should be resected completely if possible as residual HGM can produce gastric acid and induce repeated delayed hemorrhage of the wound. This is the first reported case of the resection of a large HGM lesion and a concomitant diverticulum in the rectum treated by ESD. Histological result revealed ectopic gastric mucosa of the fundic-type. No pyloric-type gastric mucosa was detected which was not in accordance with the results of NBI. This may have been due to less focal tissue of pyloric-type gastric mucosa in comparison with fundic-type gastric mucosa in the piecemeal resected lesion, the sampling or cutting site of the histological biopsy.

Recently, more and more new issues related to HGM in the rectum have been addressed. Many studies have reported the potential progression to malignancy in the esophagus and gallbladder. The risk and possibility of intestinal metaplasia, dysplasia and malignant progression to adenocarcinoma in the rectum have been incompletely evaluated. The study by Ko *et al*[21] examined a case of colonic adenocarcinoma arising from gastric heterotopia, which was the first case report in the literature. The colonization of *H. pylori* in rectal gastric heterotopic mucosa has been noted. In the review by Jarosław Swatek of 50 rectal gastric heterotopic mucosa patients, *H. pylori* was present in six patients[22]. It is of interest to note that *H. pylori* passed through the whole GI tract to the rectum. *H. pylori* infection may have resulted in chronic active gastritis, ulceration or bleeding in the rectum. Thus, in cases associated with *H. pylori*, eradication treatment should be prescribed as the first therapeutic option[23].

In summary, heterotopic gastric mucosa of the rectum is a very rare clinical entity which mainly presents as chronic painless rectal bleeding. The pathogenesis of this condition is unclear. The definitive diagnosis of HGM relies on histopathological examination. Endoscopy techniques such as NBI and CLE could help in the diagnosis of HGM without the need for biopsies. Endoscopic resection is thought to be the first-line treatment and surgical excision may be preferred in some patients. Further investigations on the pathogenesis, malignant transformation, *H. pylori* infection, and optimal treatment are necessary.

ARTICLE HIGHLIGHTS

Case characteristics
A 14-year-old girl visited our hospital due to a 5-year history of repeated hematochezia.

Clinical diagnosis
Heterotopic gastric mucosa (HGM) and a concomitant diverticulum in the rectum found through colonoscopy examination.

Differential diagnosis
Ulcerative colitis.

Laboratory diagnosis
CA 19-9 was 61.2 U/mL, hemoglobin was 116 g/L, and stool occult blood examination was weakly positive. Other laboratory results were close to normal.

Imaging diagnosis
At colonoscopy, a solitary superficial depressed lesion, approximately 5 cm in size, with a diverticular cavity was identified in the rectum. Abdominal computed tomography revealed no obvious gastrointestinal lesions.

Pathological diagnosis
The pathology test confirmed the diagnosis of HGM.

Treatment
Endoscopic submucosal dissection.

Related reports
To the best of our knowledge, this is the first report of large HGM lesion and a concomitant diverticulum in the rectum treated by endoscopic submucosal dissection (ESD).

Term explanation
HGM is the most commonly reported epithelial heterotopia and has been described as heteroplasia in organogenesis or metaplasia during the repair of damaged epithelium.

Experiences and lessons
ESD is thought to be the safe and efficient treatment for HGM.

REFERENCES

1   **Dinarvand P**, Vareedayah AA, Phillips NJ, Hachem C, Lai J. Gastric heterotopia in rectum: A literature review and its diagnostic pitfall. *SAGE Open Med Case Rep* 2017; **5**: 2050313X17693968 [PMID: 28321304 DOI: 10.1177/2050313X17693968]

2   **Salem GA**, Fazili J, Ali T. Gastric heterotopia in the rectum. A rare cause of ectopic gastric tissue. *Arab J Gastroenterol* 2017; **18**: 42-43 [PMID: 28223104 DOI: 10.1016/j.ajg.2017.01.005]

3   **Cai J**, Yu H. Giant polypoid gastric heterotopia in the small intestine in a boy: A case report and literature review. *Medicine* (Baltimore) 2017; **96**: e5854 [PMID: 28072748 DOI: 10.1097/MD.0000000000005854]

4   **Ewell GH**, Jackson RH. Aberrant gastric mucosa in the rectum with ulceration and hemorrhage. *Wis Med J* 1939; **38**: 641-643

5   **Yu L**, Yang Y, Cui L, Peng L, Sun G. Heterotopic gastric mucosa of the gastrointestinal tract: prevalence, histological features, and clinical characteristics. *Scand J Gastroenterol* 2014; **49**: 138-144 [PMID: 24279774 DOI: 10.3109/00365521.2013.860558]

6   **Huelsen A**, Falvey J, Whitehead M, Ding S. Education and Imaging. Gastrointestinal: large heterotopic gastric mucosa in the rectum. *J Gastroenterol Hepatol* 2012; **27**: 1641 [PMID: 22994433 DOI: 10.1111/j.1440-1746.2012.07224.x]

7   **Al-Hussaini A**, Lone K, Al-Sofyani M, El Bagir A. Gastric

heterotopia of rectum in a child: a mimicker of solitary rectal ulcer syndrome. *Ann Saudi Med* 2014; **34**: 245-249 [PMID: 25266186 DOI: 10.5144/0256-4947.2014.245]

8   **Wolff M**. Heterotopic gastric epithelium in the rectum: a report of three new cases with a review of 87 cases of gastric heterotopia in the alimentary canal. *Am J Clin Pathol* 1971; **55**: 604-616 [PMID: 5090217 DOI: 10.1093/ajcp/55.5.604]

9   **Ikematsu H**, Fu KI, Saito Y, Matsuda T, Shimoda T, Fujii T. Ectopic gastric mucosa in the rectum mimicking an early depressed cancer treated by endoscopic mucosal resection. *Endoscopy* 2007; **39** Suppl 1: E171-E172 [PMID: 17614073 DOI: 10.1055/s-2007-966581]

10 **Iacopini F**, Gotoda T, Elisei W, Rigato P, Montagnese F, Saito Y, Costamagna G, Iacopini G. Heterotopic gastric mucosa in the anus and rectum: first case report of endoscopic submucosal dissection and systematic review. *Gastroenterol Rep* (Oxf) 2016; **4**: 196-205 [PMID: 27103738 DOI: 10.1093/gastro/gow006]

11 **Tai CM**, Chang IW, Wang HP. Heterotopic gastric mucosa of the ileum. *Endoscopy* 2015; **47** Suppl 1: E423 [PMID: 26397851 DOI: 10.1055/s-0034-1392666]

12 **Tan Y**, Wang Y, Liu D. Heterotopic gastric mucosa mimicking a rectal submucosal tumor. *Endoscopy* 2015; **47** Suppl 1: E329-E330 [PMID: 26115400 DOI: 10.1055/s-0034-1392323]

13 **Murray FE**, Lombard M, Dervan P, Fitzgerald RJ, Crowe J. Bleeding from multifocal heterotopic gastric mucosa in the colon controlled by an H2 antagonist. *Gut* 1988; **29**: 848-851 [PMID: 3290067 DOI: 10.1136/gut.29.6.848]

14 **Limdi JK**, Sapundzieski M, Chakravarthy R, George R. Gastric heterotopia in the rectum. *Gastrointest Endosc* 2010; **72**: 190-191; discussion 191 [PMID: 20620280 DOI: 10.1016/j.gie.2010.01.045]

15 **Eguchi K**, Aoyagi K, Nimura S, Sakisaka S. Diagnostic value of endoscopic and endoscopic ultrasound characteristics of duodenal submucosal tumour-like heterotopic gastric mucosa. *Can J Gastroenterol* 2011; **25**: 365-367 [PMID: 21876857 DOI: 10.1155/2011/104815]

16 **Fang Y**, Chen L, Chen DF, Ren WY, Shen CF, Xu Y, Xia YJ, Li JW, Wang P, Zhang AR, Shao SZ, Yu XN, Peng GY, Fang DC. Prevalence, histologic and clinical characteristics of heterotopic gastric mucosa in Chinese patients. *World J Gastroenterol* 2014; **20**: 17588-17594 [PMID: 25516674 DOI: 10.3748/wjg.v20.i46.17588]

17 **Sciarra A**, Hessler R, Godat S, Fraga M, Dromain C, Duran R, Halkic N, Sempoux C. Heterotopic Gastric Mucosa in a Duplication Cyst of the Common Hepatic Duct Mimicking Cholangiocarcinoma. *Int J Surg Pathol* 2018; **26**: 84-88 [PMID: 28830294 DOI: 10.1177/1066896917727101]

18 **López-Cerón Pinilla M**, Jimeno Ramiro M, Rodríguez de Miguel C, Pellisé Urquiza M. Heterotopic gastric mucosa diagnosed by confocal endomicroscopy. *Rev Esp Enferm Dig* 2011; **103**: 40-41 [PMID: 21341938 DOI: 10.4321/S1130-01082011000100009]

19 **Sauer CG**, Bickston SJ, Borowitz SM. Gastric heterotopia of the rectum. *J Pediatr Gastroenterol Nutr* 2010; **50**: 329-333 [PMID: 19668007 DOI: 10.1097/MPG.0b013e3181a1c476]

20 **Soares J**, Ferreira C, Marques M, Corujeira S, Tavares M, Lopes J, Carneiro F, Amil Dias J, Trindade E. Endoscopic Mucosectomy in a Child Presenting with Gastric Heterotopia of the Rectum. *GE Port J Gastroenterol* 2017; **24**: 288-291 [PMID: 29255771 DOI: 10.1159/000478939]

21 **Ko H**, Park SY, Cha EJ, Sohn JS. Colonic adenocarcinoma arising from gastric heterotopia: a case study. *Korean J Pathol* 2013; **47**: 289-292 [PMID: 23837024 DOI: 10.4132/KoreanJPathol.2013.47.3.289]

22 **Swatek J**, Wronecki L, Ciechanek R, Szumiło J. Asymptomatic gastric heterotopia in the rectum with Helicobacter pylori infection. *Pol J Pathol* 2015; **66**: 426-429 [PMID: 27003777 DOI: 10.5114/pjp.2015.57258]

23 **Colsa-Gutiérrez P**, Kharazmi-Taghavi M, Sosa-Medina RD, Berrío-Obregón JI, Ingelmo-Setién A. [Heterotopic gastric mucosa in the rectum: Report of a case]. *Cir Cir* 2016; **84**: 160-163 [PMID: 26242819 DOI: 10.1016/j.circir.2015.06.013]

Baishideng®

World Journal of

Gastroenterology

Contents

Contents

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Tamara Vorobjova, DA, PhD, Academic Research, Department of Immunology, Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu 51014, Estonia

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 642 experts in gastroenterology and hepatology from 59 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

EDITORS FOR THIS ISSUE

Locoregional therapy response in patients with hepatocellular cancer waiting for liver transplantation: Only selection or biological effect?

Quirino Lai, Michele Di Martino, Pierleone Lucatelli, Gianluca Mennini

Quirino Lai, Gianluca Mennini, Department of General Surgery and Organ Transplantation, Sapienza University of Rome, Rome 00161, Italy

Michele Di Martino, Pierleone Lucatelli, Department of Radiology, Sapienza University of Rome, Rome 00161, Italy

ORCID number: Quirino Lai (0000-0003-1487-3235); Michele Di Martino (0000-0003-1504-1166); Pierleone Lucatelli (0000-0002-7448-1404); Gianluca Mennini (0000-0002-6412-6863).

Author contributions: Lai Q, Di Martino M, Lucatelli P and Mennini G conceived the study and drafted the manuscript; both authors approved the final version of the article.

Conflict-of-interest statement: The authors have no conflict of interest to declare.

Correspondence to: Quirino Lai, MD, PhD, Academic Fellow, Academic Research, Doctor, Senior Lecturer, Department of General Surgery and Organ Transplantation, Sapienza University of Rome, Viale del Policlinico 155, Rome 00161, Italy. lai.quirino@libero.it
Telephone: +39-34-93020126
Fax: +39-6-499701

Abstract

Locoregional treatments (LRT) represent a broad strategy used for reducing the risk of drop-off and contextually improving the survivals in patients with hepatocellular cancer receiving a liver transplantation (LT). However, it is not sufficiently clear if LRT are only a surrogate of tumor aggressiveness or if they consent a real benefit in terms of tumor stabilization. A recent study by Pommergaard *et al* reported the results from the European Liver Transplant Registry. Patients receiving LRT before LT had better 5-year survival rates respect to no-LRT cases (69.7% *vs* 65.8%; *P* < 0.001). When the number of LRT was tested, one-to-two treatments were connected with improved survivals respect to no treatment [hazard ratio (HR) = 0.85 and 0.71, respectively]. The efficacy of LRT was also reported in the presence of larger tumors (HR = 0.78) and micro-macrovascular invasion (HR = 0.71). The results observed in the present study are partially in discordance with other analyses showing a detrimental effect of LRT. The main problem in the interpretation of these results is connected with the possible initial selection biases present in the studies. The most recent guidelines suggest to perform LRT before the transplant, but the level of evidence is typically low due to the absence of prospectively designed studies.

Key words: Allocation; Recurrence; Trans-arterial chemo-embolization; Radiofrequency ablation; Model for end-stage liver disease

Core tip: The role of locoregional treatments in the setting of hepatocellular cancer and liver transplantation is controversial. On one side, neoadjuvant approaches should consent a selection of tumor aggressiveness. On the other side, a real survival improvement thanks to the tumor ablation should be achieved. Recent evidences report an effective beneficial role of locoregional strategies in terms of survival and recurrence. However, several biases must be taken into account in these studies, due to the heterogeneous characteristics of treated *vs* untreated subjects. Further studies are need with the intent to clarify this important topic.

INTRODUCTION

Liver transplantation (LT) represents the gold-standard treatment in patients with unresectable hepatocellular cancer (HCC) developed on underlying cirrhosis[1]. Unfortunately, LT represents a scarce resource, mainly due to the limited number of available donors[2]. Thus, in patients awaiting LT, disease burden may progress beyond the conventional LT criteria while on the waiting list[3]. Several strategies have been adopted with the intent to alleviate the risk of drop-out due to tumor progression: for example, Model for End-Stage Liver Disease (MELD) exception points are routinely used in several regions in the presence of T2 HCCs[4]. Another widespread strategy is the use of locoregional treatments (LRTs) as a neo-adjuvant strategy with the intent to bridge patients to LT[5] or downstage patients initially outside transplantation criteria[6].

Response to LRT has been correlated with improved post-LT survival rates in several studies[7,8]. However, it is not sufficiently clear if LRTs are only a surrogate of tumor aggressiveness, efficaciously selecting patients with favorable tumor biology, or if they are beneficial concerning tumor stabilization, mainly in case of complete or partial response.

STUDY ANALYSIS

In a recent Issue on Transplant International, Pommergaard *et al*[9] reported the results of a multicentric study based on the European Liver Transplant Registry (ELTR) and focused on the use of LRT in HCC patients undergoing LT. A total of 4978 patients (no LRT = 1406, 28.2%; LRT = 3572, 71.8%) were enrolled. As expected, the median waiting time was longer in the LRT group

(4 mo *vs* 1.7 mo; $P < 0.001$) and the median MELD score was higher in the directly transplanted subjects (12 *vs* 10; $P < 0.001$). Overall, patients receiving LRT before LT had better 5-year survival rates respect to no-LRT cases (69.7% *vs* 65.8%; $P < 0.001$). When the different treatment types were investigated, the use of radiofrequency ablation (RFA) had the strongest association with an improved overall survival [hazard ratio (HR) = 0.51]. The beneficial effect was also observed in case of the combination of RFA and trans-arterial chemo-embolization (TACE) (HR = 0.74). Several sub-analyses were also done. As for the number of LRT performed, one-two treatments were connected with improved survivals respect to no treatment (HR = 0.85 and 0.71, respectively). On the opposite, three or more treatments showed no association (HR = 1.11).

When a subclass of HCCs being larger (> 3 cm) or with more nodules (> 5 lesions) was examined, LRT maintained their protective role for the risk of death (HR = 0.78). In this context, RFA, TACE or combined RFA + TACE all were significantly associated with improved survival (HR = 0.54, 0.81, and 0.60, respectively).

Stratifying the entire population according to the underlying liver status (cirrhosis *vs* non-cirrhotic liver), in case of HCC on cirrhosis LRT were also protective (HR = 0.86).

In the presence of pathological micro-macrovascular invasion, the effect of LRT was strong (HR = 0.71). Both RFA and TACE (HR = 0.54 and 0.69, respectively) were associated with improved survival.

PERSPECTIVE

The role of LRT in the setting of HCC and LT has not been fully clarified, mainly in light of the potential detrimental effects of repetitive treatments. For example, a recent meta-analysis performed on 1122 TACE patients showed an increased risk of post-LT hepatic artery complications (odds ratio = 1.57; $P = 0.02$)[10]. Another study from the US performed on 3601 patients all meeting the Milan Criteria, showed that the increasing number of LRT significantly predicted post-LT recurrence (3 LRTs: HR = 2.1; $P < 0.001$; 4 + LRTs: HR = 2.5; $P < 0.001$)[5]. Interestingly, LRT patients achieving complete response had superior 5-year recurrence-free survivals when compared with untreated cases or LRT subjects not achieving complete response (72% *vs* 69% *vs* 67%; respectively)[5]. The here described study performed on a large population of European HCC cases showed the beneficial role of LRT, mainly in case of RFA use. Moreover, the repetitive number of treatments was not connected with worse results. LRT maintained their protective role for the risk of death even when larger tumors or harmful clinical conditions like vascular invasion were investigated. It is difficult to definitively clarify if the LRT only select low-risk HCC, or if their ability of tumor burden zeroing should also have some impact regarding survival improvement. It is clear that

the response after LRT is a robust predictor of post-LT course. A recent large multicentric European study based on 2103 HCC patients identified the poor radiological response after LRT as one of the most important predictors for the risk of low intention-to-treat benefit after transplant[11]. Another multicentric European study performed on 276 cases all treated with LRT showed that an HCC-related remaining vital tissue in the main lesion ≥ 2 cm at pathological assessment after LT was a strong independent risk factor for post-LT recurrence (HR = 5.6; *P* < 0.001)[12]. All of these results have been positively recognized by the recent European Association for the Study of the Liver (EASL) guidelines, in which it is stated that "in LT candidates with HCC, the use of pre-transplant (neoadjuvant) loco-regional therapies is recommended if feasible, as it reduces the risk of pre-LT drop-out and aims at lowering post-LT recurrence - particularly when complete or partial tumour response are achieved"[13]. Unfortunately, although the strength of recommendation for this statement is strong, the scientific evidence is low, clearly underlining the lack of prospectively designed studies. More researchers are needed, with the intent to better explore the role of LRT concerning intention-to-treat survivals.

Moreover, we should remember the critical impact that local allocation rules and waiting time duration may play on the role and the effect of LRT. As an example, in the United States most HCC patients wait for at least six months from the diagnosis before having the opportunity to be transplanted. More studies also focused on these aspects are surely needed.

REFERENCES

1 **Lai Q**, Lerut JP. Hepatocellular cancer: how to expand safely inclusion criteria for liver transplantation. *Curr Opin Organ Transplant* 2014; **19**: 229-234 [PMID: 24811435 DOI: 10.1097/MOT.0000000000000085]

2 **Lai Q**, Melandro F, Levi Sandri GB, Mennini G, Corradini SG, Merli M, Berloco PB, Rossi M. Use of elderly donors for liver transplantation: has the limit been reached? *J Gastrointestin Liver Dis* 2011; **20**: 383-387 [PMID: 22187704]

3 **Mehta N**, Heimbach J, Lee D, Dodge JL, Harnois D, Burns J, Sanchez W, Roberts JP, Yao FY. Wait Time of Less Than 6 and Greater Than 18 Months Predicts Hepatocellular Carcinoma Recurrence After Liver Transplantation: Proposing a Wait Time "Sweet Spot". *Transplantation* 2017; **101**: 2071-2078 [PMID: 28353492 DOI: 10.1097/TP.0000000000001752]

4 **Marrero JA**, Kulik LM, Sirlin C, Zhu AX, Finn RS, Abecassis MM, Roberts LR, Heimbach JK. Diagnosis, Staging and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. *Hepatology* 2018; Epub ahead of print [PMID: 29624699 DOI: 10.1002/hep.29913]

5 **Agopian VG**, Harlander-Locke MP, Ruiz RM, Klintmalm GB, Senguttuvan S, Florman SS, Haydel B, Hoteit M, Levine MH, Lee DD, Taner CB, Verna EC, Halazun KJ, Abdelmessih R, Tevar AD, Humar A, Aucejo F, Chapman WC, Vachharajani N, Nguyen MH, Melcher ML, Nydam TL, Mobley C, Ghobrial RM, Amundsen B, Markmann JF, Langnas AN, Carney CA, Berumen J, Hemming AW, Sudan DL, Hong JC, Kim J, Zimmerman MA, Rana A, Kueht ML, Jones CM, Fishbein TM, Busuttil RW. Impact of Pretransplant Bridging Locoregional Therapy for Patients With Hepatocellular Carcinoma Within Milan Criteria Undergoing Liver Transplantation: Analysis of 3601 Patients From the US Multicenter HCC Transplant Consortium. *Ann Surg* 2017; **266**: 525-535 [PMID: 28654545 DOI: 10.1097/SLA.0000000000002381]

6 **Kulik L**, Heimbach JK, Zaiem F, Almasri J, Prokop LJ, Wang Z, Murad MH, Mohammed K. Therapies for patients with hepatocellular carcinoma awaiting liver transplantation: A systematic review and meta-analysis. *Hepatology* 2018; **67**: 381-400 [PMID: 28859222 DOI: 10.1002/hep.29485]

7 **Lai Q**, Nicolini D, Inostroza Nunez M, Iesari S, Goffette P, Agostini A, Giovagnoni A, Vivarelli M, Lerut J. A Novel Prognostic Index in Patients With Hepatocellular Cancer Waiting for Liver Transplantation: Time-Radiological-response-Alpha-fetoprotein-INflammation (TRAIN) Score. *Ann Surg* 2016; **264**: 787-796 [PMID: 27429025 DOI: 10.1097/SLA.0000000000001881]

8 **Lai Q**, Avolio AW, Graziadei I, Otto G, Rossi M, Tisone G, Goffette P, Vogel W, Pitton MB, Lerut J; European Hepatocellular Cancer Liver Transplant Study Group. Alpha-fetoprotein and modified response evaluation criteria in solid tumors progression after locoregional therapy as predictors of hepatocellular cancer recurrence and death after transplantation. *Liver Transpl* 2013; **19**: 1108-1118 [PMID: 23873764 DOI: 10.1002/lt.23706]

9 **Pommergaard HC**, Rostved AA, Adam R, Thygesen LC, Salizzoni M, Gómez Bravo MA, Cherqui D, De Simone P, Boudjema K, Mazzaferro V, Soubrane O, García-Valdecasas JC, Fabregat Prous J, Pinna AD, O'Grady J, Karam V, Duvoux C, Rasmussen A; European Liver and Intestine Transplant Association (ELITA). Locoregional treatments before liver transplantation for hepatocellular carcinoma: a study from the European Liver Transplant Registry. *Transpl Int* 2018; **31**: 531-539 [PMID: 29380442 DOI: 10.1111/tri.13123]

10 **Sneiders D**, Houwen T, Pengel LHM, Polak WG, Dor FJMF, Hartog H. Systematic Review and Meta-Analysis of Posttransplant Hepatic Artery and Biliary Complications in Patients Treated With Transarterial Chemoembolization Before Liver Transplantation. *Transplantation* 2018; **102**: 88-96 [PMID: 28885493 DOI: 10.1097/TP.0000000000001936]

11 **Lai Q**, Vitale A, Iesari S, Finkenstedt A, Mennini G, Spoletini G, Hoppe-Lotichius M, Vennarecci G, Manzia TM, Nicolini D, Avolio AW, Frigo AC, Graziadei I, Rossi M, Tsochatzis E, Otto G, Ettorre GM, Tisone G, Vivarelli M, Agnes S, Cillo U, Lerut J; European Hepatocellular Cancer Liver Transplant Study Group. Intention-to-treat survival benefit of liver transplantation in patients with hepatocellular cancer. *Hepatology* 2017; **66**: 1910-1919 [PMID: 28653750 DOI: 10.1002/hep.29342]

12 **Manzia TM**, Lai Q, Iesari S, Perera MTPR, Komuta M, Carvalheiro A, Shah T, Angelico R, Quaranta C, Nicolini D, Montalti R, Scarpelli M, Palmieri G, Orlacchio A, Vivarelli M, Angelico M, Lerut J, Tisone G. Impact of remnant vital tissue after locoregional treatment and liver transplant in hepatocellular cancer patients, a multicentre cohort study. *Transpl Int* 2018; Epub ahead of print [PMID: 29572974 DOI: 10.1111/tri.13153]

13 **European Association for the Study of the Liver**; European Association for the Study of the Liver. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol* 2018; **69**: 182-236 [PMID: 29628281 DOI: 10.1016/j.jhep.2018.03.019]

REVIEW

Helicobacter pylori: A foodborne pathogen?

Nicoletta C Quaglia, Angela Dambrosio

Nicoletta C Quaglia, Angela Dambrosio, Department of Emergency and Organ Transplantation, Section of Veterinary Clinic and Animal Production, University of Bari "Aldo Moro", Valenzano 70010, Italy

ORCID number: Nicoletta C Quaglia (0000-0003-4854-7390); Angela Dambrosio (0000-0002-4935-4331).

Abstract

Helicobacter pylori (*H. pylori*) is an organism that is widespread in the human population and is sometimes responsible for some of the most common chronic clinical disorders of the upper gastrointestinal tract in humans, such as chronic-active gastritis, duodenal and gastric ulcer disease, low-grade B-cell mucosa associated lymphoid tissue lymphoma of the stomach, and gastric adenocarcinoma, which is the third leading cause of cancer death worldwide. The routes of infection have not yet been firmly established, and different routes of transmission have been suggested, although the most commonly accepted hypothesis is that infection takes place through the faecal-oral route and that contaminated water and foods might play an important role in transmission of the microorganism to humans. Furthermore, several authors have considered *H. pylori* to be a foodborne pathogen because of some of its microbiological and epidemiological characteristics. *H. pylori* has been detected in drinking water, seawater, vegetables and foods of animal origin. *H. pylori* survives in complex foodstuffs such as milk, vegetables and ready-to-eat foods. This review article presents an overview of the present knowledge on the microbiological aspects in terms of phenotypic characteristics and growth requirements of *H. pylori*, focusing on the potential role that foodstuffs and water may play in the transmission of the pathogen to humans and the methods successfully used for the detection of this microorganism in foodstuffs and water.

Key words: *Helicobacter pylori*; Viable but nonculturable state; Foodborne pathogen; Food; Water; Animal reservoirs; Culture methods; Molecular methods

Core tip: To date, the transmission routes and reservoirs of *Helicobacter pylori* (*H. pylori*) are topics of debate. Epidemiological evidence and the occurrence of *H. pylori* in foods of animal origin, vegetables and water corroborate the hypothesis advanced by numerous authors that *H. pylori* may be a foodborne pathogen. The present review is focused on the

evidence supporting the role of foods and water in the transmission of *H. pylori* to humans and on the methods for detecting the pathogen in foodstuffs and water.

INTRODUCTION

The first isolation of *Helicobacter pylori* (*H. pylori*) in 1982 by Marshall and Warren[1,2] marked a turning point in understanding gastrointestinal microbial ecology and disease[3]. Following the initial scepticism regarding the aetiologic importance of this organism, it is now recognized that infections with *H. pylori* are linked to some of the most common chronic clinical disorders of the upper gastrointestinal tract in humans[4]. In fact, *H. pylori* has been acknowledged as a major cause of chronic-active gastritis and is associated with duodenal and gastric ulcer disease, low-grade B-cell mucosa-associated lymphoid tissue lymphoma of the stomach (MALToma)[5], and gastric adenocarcinoma, which is the third leading cause of cancer death worldwide[4,6,7]. Furthermore, *H. pylori* has been linked to a variety of extra-gastric disorders, including coronary heart disease, dermatological disorders such as rosacea and idiopathic urticaria, autoimmune thyroid disease, thrombocytopenic purpura, and iron deficiency anaemia[8].

Human infection by *H. pylori* is a great public health hazard because *H. pylori* colonizes the gastric mucosa of approximately half of the world's population[9-12]. The infection is usually acquired in infancy and early childhood, and it is long lasting, often remaining for the entire lifespan[13]. The prevalence of *H. pylori* shows large geographical variation, with infection rates much higher in developing countries (in some areas > 85%) than in Europe and North America (approximately 30%-40%)[14,15]. In various developing countries, more than 80% of the population is *H. pylori* positive, even at young ages[16]. The prevalence of *H. pylori* in industrialized countries generally remains under 40% and is considerably lower in children and adolescents than in adults and elderly people. A comparison of prevalence rates by age suggests that the acquisition of *H. pylori* is decreasing in recent cohorts, and this finding is most apparent in developed countries and may be linked to improvements in hygiene practices. Furthermore, it has been estimated that between two and 20 percent of people infected with *H. pylori* develop peptic ulcer disease[17].

Although *H. pylori* can cause severe illnesses with a high rate of morbidity and mortality, the complex interactions between this microbe and humans,

particularly its transmission pathways to humans and reservoirs, are largely unknown, although multiple routes of transmission have been suggested[3,18-20]. The current literature suggests that the transmission of *H. pylori* occurs from person to person *via* the oral-oral, faecal-oral, and gastric-oral routes and that the infection dose for humans is low[6,21]. *H. pylori* may be a sex-transmitted pathogen[22,23] and may lead to fibrocystic breast changes[24]. The oral cavity can be primarily colonized by *H. pylori*, and this can be linked to later gastric infection[25]. Faecal-oral transmission has more important implications than oral-oral transmission because *H. pylori* may occur in food and water supplies subsequent to faecal contamination[26]. Furthermore, several authors have considered *H. pylori* to be a foodborne pathogen because of its microbiological and epidemiological characteristics[6,10,27-31]. Information on the distribution of *H. pylori* in water, vegetables and foods of animal origin is critical in determining its potential transmission in foods.

This review article presents a brief overview of the present knowledge on the microbiological characteristics of *H. pylori* in terms of its phenotypic characteristics and growth requirements, focusing on the potential role that foodstuffs and water may play in the transmission of this pathogen to humans and the methods for isolating and detecting this microorganism in foodstuffs and water.

LITERATURE SEARCH

A PubMed search was conducted using the following keywords and phrases: "*Helicobacter pylori*, *Helicobacter pylori* and food, *Helicobacter pylori* and milk, *Helicobacter pylori* and water, VBNC, survival of *Helicobacter pylori*". In addition, we performed a manual review of the reference lists of the primary and review articles to ensure identification of all relevant articles.

MICROBIOLOGICAL CHARACTERISTICS

Phenotypic characteristics

H. pylori was originally thought to be a species belonging to the genus *Campylobacter* and was first named *Campylobacter pyloridis*, which was later corrected to *Campylobacter pylori* (*C. pylori*)[32]. Because subsequent 16S rRNA sequence analysis showed that the distance between the species belonging to the genus *Campylobacter* and *C. pylori* was sufficient to exclude *C. pylori* from this genus[33], it was renamed *Helicobacter pylori*[34], the first member of the new genus *Helicobacter*.

H. pylori organisms are spiral or curved bacilli ranging from 0.3 to 1.0 μm in width and 1.5 to 10.0 μm in length; they are gram-negative and assume a rod-like shape when cultured on solid medium[34]. Furthermore, after prolonged *in vitro* culture and under adverse environmental conditions, such as an insufficient supply of nutrients, desiccation, lack of protection against oxygen, and exposure to antimicrobial agents, *H. pylori* can survive entering the viable but non-culturable

(VBNC) state, changing its rod-like shape to a coccoid shape[35-37].

When this morphological change occurs, *H. pylori* is unable to grow on agar plates using conventional cultivation methods[38-40].

Bacteria in the VBNC state maintain their metabolic activity, pathogenicity and ability to return to active regrowth conditions[41,42]. For *H. pylori,* the ability to return to active regrowth conditions has not yet been proven. Nevertheless, the aptitude of *H. pylori* to overcome stressed conditions is very significant for public health[29], even if the role of VBNC in the transmission of *H. pylori*, especially by food and water, is still controversial.

H. pylori is motile and usually possesses four to six unipolar-sheathed flagella, which may be an adaptation to survive in gastric juices[3,43].

Growth requirements

Since the discovery of *H. pylori*, bacterial culture has been used as a routine diagnostic test and is considered the gold standard. *H. pylori* culture is recommended for performing antibiotic susceptibility testing if primary resistance to clarithromycin is higher than 20% or after failure of second-line treatment[44]. Despite the long use of bacterial culture, to date, there are no defined media for the selective culture of *H. pylori* because of its fastidious nature with particular growth requirements of atmosphere, nutrient-rich media, high humidity (98%), and long incubation time (5-7 d)[3,44].

H. pylori is a capnophilic organism that requires an atmosphere with a high level of CO_2 (from 5% to 10%). It has been considered a microaerophile, but the concentration of O_2 required for its growth is still a topic of discussion[44].

H. pylori requires a complex culture substrate (solid or liquid) with some forms of supplementation, such as whole sheep or horse blood, haemoglobin, serum, coal, yeast, or yolk emulsion[45,46], which may serve as nutritional substrates. These supplements also detoxify the medium and protect the microorganism[7].

Furthermore, if isolation is attempted from samples with basic microbial flora, it is necessary to make the media selective through supplementation of several antibiotics[47].

Growth in liquid media is enhanced by agitation, which allows gas dispersion and incubation in a CO_2-rich atmosphere[31,47].

H. pylori grows within a temperature range of 30 ℃ to 37 ℃, with optimum growth at 37 ℃, but is not able to grow at 25 ℃[43]. At 42 ℃, growth is variable[29].

Similar to *C. jejuni*, *H. pylori* survives longer at 4 ℃ than at room temperature, and it grows within a pH range of 4.5 to 7.3, with optimum at pH 5.5. *H. pylori* grows well in the presence of 0.5% and 1% NaCl but not of 2% NaCl. The minimum water activity (a_w) for growth is between 0.96 and 0.98. These data suggest that this microorganism is most likely not able to grow in many types of food[47].

H. pylori is catalase and oxidase positive; it is also characterized by strong urease activity and is negative for hippurate and nitrate reduction, characteristics that discriminate it from species belonging to the genus *Campylobacter*[29].

EVIDENCE SUPPORTING THE ROLE OF FOODS IN TRANSMISSION OF *H. PYLORI* TO HUMANS

Since 1997, when the transmission of *H. pylori* through water and foods was hypothesized for the first time, several studies have evaluated the survival and the presence of this microorganism in different foodstuffs (Tables 1 and 2).

One of the most important topics supporting this thesis was the phylogenetic proximity of *H. pylori* to *C. jejuni*, which led to the hypothesis that the transmission pathways described for the latter could also be applied to *H. pylori*[27]. Several investigators have also considered *H. pylori* a foodborne pathogen based on some of its epidemiological characteristics[27,28,30] such as the high prevalence of infection within closed family groups and among individuals living in institutions[48]. These aspects suggest that in addition to direct transmission, this bacterium may be transmitted indirectly through a common source, such as through consumption of the "same foods at the same table"[26]. The finding that the prevalence of *H. pylori* infection is greater in geographical areas in which the hygienic conditions of life are poor also supports this hypothesis[30,49].

Additional indirect evidence of the transmission of *H. pylori* to humans through foods of animal origin has been provided by epidemiological studies on the presence of antibodies in slaughterhouse workers and in veterinary workers. The incidence rates in these workers were positive and were greater than those in workers who had no direct contact with carcasses[50,51] (Figure 1).

Foods presenting intrinsic factors, such as a_w higher than 0.97 and pH ranging from 4.9 to 6.0, could theoretically provide conditions for *H. pylori* survival[30,52].

Therefore, data on survival ability may be more important than concerns about the growth of the microorganism in foods when determining the role of foods in *H. pylori* transmission to humans[53].

Survival of H. pylori in foodstuffs

Several studies have demonstrated the survival of *H. pylori* in water, milk, ready-to-eat foods, vegetables, pasteurized apple and orange juices, ground beef and dry fermented sausages[26,53-60] (Table 1).

H. pylori is able to survive in artificially contaminated milk stored at 4 ℃ for several days (from 5 to 9 d in pasteurized milk and from approximately 6 to 12 d in sterile milk)[26,53,54,56]. These findings corroborate the hypothesis that post-processed contaminated milk may play a more effective role than other foods in the transmission of *H. pylori* infection due to the intrinsic

Table 1 Studies evaluating the survival of *Helicobacter pylori* in artificially contaminated foods

Year	Food	Method	Observations	Ref.
1998	Sterilized milk	Bacterial count on chocolate agar	10 d at 4 ℃ 3 d at 25 ℃	Fan *et al*[54]
2001	Pasteurized milk water tofu, tofu, yogurt, lettuce and chicken	Bacterial count on tryptic soy agar, non-selective Wilkins-Chalgren Anaerobe blood agar and selective Wilkins-Chalgren Anaerobe blood agar	from 5 to 7 d in pasteurized milk, tofu and water tofu at 4 ℃ for up to 2 d in lettuce and raw chicken at 4 ℃ for up to 1 d in yogurt at 4 ℃	Poms *et al*[26]
2000	Ground beef packaged in vacuum and air	Bacterial count on *H. pylori* special peptone agar	6 d in ground beef packaged in air at 4 ℃ 3-6 d in ground beef packaged in vacuum at 4 ℃ 3 d in ground beef packaged in air and in vacuum at -18 ℃	Stevenson *et al*[55]
2002	Ground beef, sterile milk, and apple and orange juices	Bacterial count on brain heart infusion agar and horse serum	7 d in ground beef at 4 ℃ 11 d in irradiated ground beef at 4 ℃ 6 d in sterile milk at 4 ℃ 1 d in apple and orange juice at 4 ℃ and 25 ℃	Jiang *et al*[56]
2004	Lettuce and carrots	Bacterial count on *Helicobacter* special peptone agar and Columbia blood agar	3 d in lettuce at 8 ℃ 5 d in sterilized carrot at 8 ℃ 3 d in sanitized carrot at 8 ℃	Gomes *et al*[57]
2007	Sterile milk and pasteurized milk	Bacterial count on Wilkins-Chalgren anaerobe agar	12 d in sterile milk at 4 ℃ 9 d in pasteurized milk at 4 ℃	Quaglia *et al*[53]
2010	Spinach	Bacterial count on brucella blood agar, Wilkins-Chalgren anaerobe blood agar	6 d at 8 ℃	Buck *et al*[58]
2011	Traditional Turkish fermented sausage (*sucuk*)	Bacterial count on Wilkins-Chalgren anaerobe blood agar	7 d	Guner *et al*[59]
2017	Spring onion, cabbage, lettuce and spinach	Bacterial count on non-selective Blood base agar with 5% horse blood	3 d in spring onion, lettuce and spinach 4 d in cabbage stored at 4 ℃	Ng *et al*[60]

characteristics of this organism[53]. It is well known that *H. pylori*'s ability to survive in an acidic pH environment is urea dependent[3], and because urea is present in milk[61], the urea-dependent acid resistance of *H. pylori* may account for the long-term survival of *H. pylori* in this foodstuff[47]. Moreover, the microorganism is able to survive in milk for longer than the best-before date on an open milk package, making milk a possible source of transmission of this microorganism to humans. In fact, although the *H. pylori* load contaminating milk under natural conditions is unknown (although it is presumably lower than that used *in vitro*), the infection dose for humans is low; thus, even a small number of *H. pylori* cells surviving in foods may represent a potential health hazard for consumers[53].

Other studies have been conducted on the survival of microorganisms in other more complex foodstuffs. *H. pylori* survives for approximately 7 d in ground beef at 4 ℃, up to 3 d at -18 ℃[55,56] and for only 2 d in prepacked boneless, skinless chicken thighs[26]. Vacuum packaging has no impact on survival time. However, if the high level of background bacteria present in the ground beef is eliminated, survival time increases to an undetectable level (< 10 cfu/g) within 11 d[55]. The fate of *H. pylori* during the fermentation process of a traditional Turkish fermented sausage (*sucuk*) was investigated. The results of this study showed that the microorganism could survive and grow during the

fermentation process of *sucuk* (22 ℃ for 7 d). A possible explanation is that some fermentation products, such as protein degradation compounds and CO_2, might have been used by this pathogen and that indigenous bacteria might have created a microenvironment suitable for *H. pylori* growth[59]. In contrast, *H. pylori* is not able to survive in yogurt[26] or pasteurized fruit juice[56] because its growth is hampered by the acidic pH and organic acids from lactic acid bacteria growth[62,63].

Survival time in vegetables is shorter: 3 d in sanitized lettuce and carrot stored at 8 ℃, 4 d in sterilized carrot and 5 d in carrot packaged in a modified atmosphere[57]. A possible explanation could be the not-robust nature of this bacterium that on the surface of vegetables is exposed to oxygen and desiccation as opposed to what happens in liquid food and the presence of a high load of natural bacterial flora. Moreover, *H. pylori* is able to survive in contaminated vegetables despite the abovementioned adverse conditions, as it is able to form biofilms[60]. However, a study on the survival of *H. pylori* in artificially contaminated spinach showed that this bacterium is able to survive for up to 6 d in VBNC forms that are still viable and can maintain its virulence factors despite its lack of cultivability[58].

Occurrence of H. pylori in foodstuffs
Based on these findings, several studies have attempted to prove the occurrence of *H. pylori* in foodstuffs (Table

Figure 1 Evidence supporting the role of foods and water in the transmission of *Helicobacter pylori* to humans. In light blue is the epidemiological evidence supporting the hypothesis that *H. pylori* is a foodborne and/or a waterborne pathogen; in orange are the risks factors for *H. pylori* infection. *H. pylori*: *Helicobacter pylori*.

2). To the best of our knowledge, the first report about the presence of *H. pylori* in sheep milk was prompted by the observation that Sardinian shepherds with direct animal contact had a higher prevalence of infection than did their same-household siblings[64]. *H. pylori* was isolated in 1 out of 38 PCR-positive raw sheep milk samples and in one out of 6 PCR-positive sheep gastric tissue samples[65].

After these findings, *H. pylori* has rarely been isolated from raw milk samples[66-69]. Bacteriological isolation of *H. pylori* occurred in one sample of raw cow milk out of 13 PCR-positive samples during a survey conducted in Japan[66]. It was also isolated in 4 samples of raw cow milk out of 20 samples analysed in Greece[67] and in Iran in 2 samples of raw sheep milk out of 11 PCR-positive samples and in 1 raw buffalo milk out of 15 PCR-positive samples[68]. Afterward, Mousavi *et al*[69] and Saedi *et al*[70] reported a higher prevalence of *H. pylori* in raw cow, sheep, goat, buffalo and camel milks in Iran than that previously mentioned.

Furthermore, only a few studies have been carried out on the occurrence of *H. pylori* in dairy products other than milk. For example, in the survey of Mousavi *et al*[69], 30% of Iranian traditional cheese, 15% of cream, 5% of butter and 27% of ice cream samples all made from unpasteurized milk were positive for *H. pylori*.

Compared to the few bacteriological isolations, the prevalence of *H. pylori* DNA is higher depending on the sensitivity of the method employed and the target gene[65,66,68,71-73]. Conversely, these findings were not confirmed in the studies conducted by Jiang and Doyle[56], Turutoglu *et al*[74] and Bianchini *et al*[75], which failed to detect *H. pylori* in cow and sheep raw milk in the United States, Turkey and Italy, respectively, by PCR and bacteriological analysis.

The attempts to culture *H. pylori* from the majority of PCR-positive samples may have been unsuccessful for several reasons: the low number of contaminating bacteria in milk samples, the presence of VBNC forms that are not detectable by conventional microbiological culture-based protocols, and the relatively long period of storage before analysis, which could have affected the vitality of the few *H. pylori* cells present in the contaminated samples[37,67,71].

Studies on the detection of *H. pylori* in food products other than milk are quite rare. *H. pylori* was isolated in 25%, 37%, 22%, 28% and 14% of cow, sheep, goat, buffalo and camel meat samples[70] and in 1.42% and 12.5% of hamburger and minced beef samples, respectively[76]. *H. pylori* DNA was detected in 36% and 44% of raw chicken and ready-to-eat raw tuna meat samples, respectively[77]. Furthermore, Hemmatinezhad *et al*[78] analysed 550 samples of ready-to-eat foods, detecting *H. pylori* in 74% of samples; olive salad (36%), restaurant salad (30%), fruit salad (28%) and soup (22%) were the most commonly contaminated. Additionally, Ghorbani *et al*[79] recovered *H. pylori* in 60 out of 300 ready-to-eat food samples (20%), including ready-to-eat fish (15%), ham (8.33%), chicken sandwiches (5%), vegetable sandwiches (18%), meat sandwiches (10%), and minced meat (32%).

Few reports have addressed the occurrence of *H. pylori* in vegetables. In two surveys conducted in Iran, many of the vegetables analysed were positive for the presence of this microorganism: 13.72%[80]

Table 2 Studies evaluating the occurrence of *Helicobacter pylori* in foods

Year	Food	Method	Number of samples *n*	Observations	Ref.
2001	Raw sheep milk	Culture and PCR	63 raw sheep milk	60% PCR positive samples 2.6% culture positive samples	Dore *et al*[65]
2002	Raw and pasteurized cow milk	Semi nested PCR, culture method and electron microscopy	18 raw cow milk	Raw milk: 72.2% semi-nested PCR positive samples; 1 culture positive sample	Fujimura *et al*[66]
			20 pasteurized milk	Pasteurized milk: 55% semi-nested PCR positive samples	
2002	Raw sheep milk	Culture	440 raw sheep milk	0% positive samples	Turutoglu *et al*[74]
2008	Raw goat, sheep, and cow milks	Nested-PCR	160 raw goat milk	25.6% positive goat milk	Quaglia *et al*[71]
			130 raw sheep milk	33% positive sheep milk	
			110 raw cow milk	50% positive cow milk	
2008	Raw chicken and ready-to-eat raw tuna	Multiplex PCR	11 raw chicken	36% positive raw chicken	Meng *et al*[77]
			18 ready-to-eat raw tuna	44% positive ready-to-eat raw tuna	
2011	Raw cow milk	FISH	20	20% positive samples	Angelidis *et al*[67]
2012	Raw cow, sheep, goat, buffalo and camel milks	PCR	75 raw cow milk	16.00% positive cow milk	Rahimi *et al*[68]
			58 raw sheep milk	13.79% positive sheep milk	
			42 raw goat milk	4.76% positive goat milk	
			20 raw buffalo milk	13.33% positive camel milk	
			15 raw camel milk	20.00% positive buffalo milk	
2014	Milk and traditional dairy products	Culture and PCR	120 raw cow milk	16.6% positive cow milk	Mousavi *et al*[69]
			100 raw goat milk	28% positive goat milk	
			100 raw sheep milk	35% positive sheep milk	
			80 raw buffalo milk	15% positive buffalo milk	
			60 raw camel milk	13.3% positive camel milk	
			60 raw donkey milk	0% positive donkey milk	
			100 cheese	30% positive cheese	
			100 butter	15% positive cream	
			100 cream	5% positive butter	
			100 ice cream	27% positive ice cream	
2014	Vegetables and salad	Culture and PCR	60 salad	16.6% positive salad	Atapoor *et al*[81]
			40 basil	12.5% positive basil	
			40 radish	7.5% positive radish	
			40 leek	20% positive leek	
			80 spinach	6.25% positive spinach	
			80 lettuce	13.75% positive lettuce	
			120 parsley	6.6% positive parsley	
2014	Washed and unwashed vegetables	Culture and PCR	430 washed and unwashed vegetable	13.72% positive vegetables and salads	Yahaghi *et al*[80]
2015	Raw cow, sheep, goat, buffalo and camel milks	PCR	75 raw cow milk	16.00% positive cow milk	Talaei *et al*[72]
			58 raw sheep milk	13.79% positive sheep milk	
			42 raw goat milk	4.76% positive goat milk	
			20 raw buffalo milk	13.33% positive camel milk	
			15 raw camel milk	20.00% positive buffalo milk	
2015	Raw cow milk	Culture and nested PCR	50 raw cow milk	22% positive cow milk	Osman *et al*[73]
2015	Raw cow milk	Culture and nested PCR	163 raw cow milk	0% positive cow milk	Bianchini *et al*[75]
2016	Raw cow, sheep, goat, buffalo and camel milks and meats	Culture and PCR	420 raw milk	21.90% positive raw milk	Saedi *et al*[70]
			400 raw meat	26.25% positive meat	
2016	Ready-to-eat food	Culture and PCR	550 ready-to-eat food	13.45% positive ready-to-eat food	Hemmatinezhad *et al*[78]
2016	Ready-to-eat food and minced meat	Culture and PCR	60 ready-to-eat fish	15% positive ready-to-eat fish	Ghorbani *et al*[79]
			60 ham	8.33% positive ham	
			40 chicken sandwich	5% positive chicken sandwich	
			40 vegetable sandwich	45% positive vegetable sandwich	
			50 meat sandwich	20% positive meat sandwich	
			50 minced meat	32% positive minced meat	
2017	Hamburger and minced meat	Culture and nested PCR	80 hamburger	1.42% positive hamburger	Gilani *et al*[76]
			70 minced-meat	12.5% positive minced-meat	

and 9.56%[81] of the vegetables and traditional salads analysed.

The high prevalence of *H. pylori* in ready-to-eat foods, meats, milks and vegetables could be due to post-

processing contamination. In fact, the high prevalence of *H. pylori* in healthy human carriers[11] suggests that foodstuff contamination due to poor hygiene management during milking, chilling and storage and during the handling, preparation and packaging of ready-to-eat foods may occur. Furthermore, *H. pylori* strains isolated from foods showed genotypes of *vac*A alleles similar to those in isolates from human clinical samples, endorsing the hypothesis that foods can be the source of *H. pylori* transmission to humans[76,78-80].

However, the existence of animal reservoirs of the microorganism cannot be excluded[65,66]. In addition, the histopathology of lesions by *H. pylori* in humans differs from that of many other gastric helicobacters, causing mild or absent inflammatory responses in their natural hosts[82]. These data suggest that *H. pylori* may have not originally evolved as a human pathogen but was likely introduced into the human population from a mammalian reservoir sometime in the distant past[65]. This hypothesis is further supported by evidence of *H. pylori* in the gastric mucosa of calves, pigs and horses[83] and its isolation from sheep gastric tissue and milk[65,84]. Furthermore, in the studies of Papiez *et al*[85] and Dore *et al*[65], *H. pylori* prevalence was higher in shepherds with direct animal contact than in controls without contact with sheep. Considering the 100% positive 13C-urea breath test in sheep, it may be reasonable to assume that these animal species may act as reservoirs and spreaders of *H. pylori*[85,86]. However, further epidemiological and experimental studies are needed to corroborate these few data.

EVIDENCE SUPPORTING THE ROLE OF WATER IN THE TRANSMISSION OF *H. PYLORI* TO HUMANS

In the last Joint Monitoring Report (JMP) of 2017, "Progress on Drinking Water, Sanitation and Hygiene" by WHO and UNICEF[87], the first global assessment of safe drinking water and sanitation services was reported. In 2015, approximately 2.1 billion people did not manage water safely, and among them, 844 million did not even have basic drinking water services, spending more than 30 min per trip to collect water from external sources, and some of them still drank untreated water from surface water sources such as streams and lakes. Globally, at least 2 billion people use a stool-contaminated source of drinking water. Contaminated water can transmit diseases such as diarrhoea, cholera, dysentery, typhoid and polio[87].

It has been estimated that *H. pylori* colonizes more than half of the world's population, and contaminated water is mentioned as one of major causes[60,88-91].

Bellack *et al*[92] developed a conceptual model of the role of water in *H. pylori* transmission. The hypothesis is that both humans and animals are long-term hosts and that water is a relatively short-term reservoir. *H. pylori*

may survive in water for a period before it is ingested as drinking water, accidentally during bathing, or through other pathways involving food. The infected person will spread *H. pylori* through faeces; through direct faecal-oral transmission, an infected person can infect another person or contaminate water bodies through direct contamination with faeces or indirectly with wastewater that comes into contact with the water used to drink. Animal contamination of water reserves may occur by defecating directly in surface waters or by faeces penetrating groundwater. The type of soil and heavy rain events can play an important role because they can facilitate the penetrability of manure containing bacteria in groundwater[92].

Several epidemiological studies have been conducted on the transmission of *H. pylori* through water, and several risk factors have been highlighted, such as living in a house with a lack of internal water pipes, the use of well or river water, the use of a latrine, less frequent boiling of drinking water and little hand washing after use of the bath and before meals[93-96] (Figure 1).

In 1991, a survey carried out on 407 Peruvian Lima children aged 2 mo to 12 years from families with different socioeconomic statuses showed an overall *H. pylori* prevalence of 48%. The children underwent the 13C-urea breath test, and the results showed a higher incidence among the children of low-income families than among those of high-income families (56% *vs* 32%). An important risk factor was the water supply; incidence increased three-fold when the water sources were outside the home compared to those whose homes had internal water sources. Furthermore, the municipal water supply seemed to be an important source of infection among Lima children from families of both low and high socioeconomic status because children from high-income families whose homes had municipal water were 12 times more likely to be infected than were those from high-income families whose water supplies came from community wells[97].

The poor basic hygiene conditions and the lack of potable water have been reported as the cause of *H. pylori* infection in an epidemiological study of a population in a rural area of the state of Mato Grosso in Brazil. The survey was conducted on 40 children and adolescents and 164 adults. *H. pylori* antibodies were detected in 31 (77.5%) children and adolescents and in 139 (84.7%) adults. The most important identified risk factor is using untreated water that could be contaminated by wastewater due to the lack of a sewage system[98].

Nurgalieva *et al*[49] conducted a similar study on 233 adults and 55 children in Kazakhstan. The overall prevalence of *H. pylori* was 86% among adults and 64% among children. The prevalence of *H. pylori* infection was inversely correlated with the index of clean water (CWI) (boil water before consumption, frequency of recovery and reuse of water and frequency of bath and shower). Infection was significantly lower among those

with a high CWI (56%) than those with a moderate (79%) or low (95%) CWI. Moreover, the prevalence of *H. pylori* was inversely related to socioeconomic status. Those living in a family in which the levels of education and study were low had a higher rate of *H. pylori* infection (90%) than did those from a higher socioeconomic group (69%)[49].

In another epidemiological survey conducted in Germany on 3347 children from cities and rural areas, 179 children (119 from cities and 60 from rural areas) were infected by *H. pylori*. Among the children from rural areas, positivity significantly increased with the consumption of water from non-municipal sources[99].

Fujimura *et al*[100] studied the presence of *H. pylori* in 4 Japanese rivers and in 224 children who lived near one river using the stool antigen test for *H. pylori* prevalence.

The results of this study showed that *H. pylori* DNA was frequently present in river water from the middle and downstream reaches in which the human biosphere is embedded. The author concluded that river water in the natural environment could be a risk factor for *H. pylori* transmission.

Occurrence of H. pylori in water

Despite the several epidemiological studies that support the hypothesis that *H. pylori* is a waterborne pathogen, the real role of water in the spread of the pathogen remains a topic of discussion. As with foodstuffs, the fastidious nature of the bacterium and the difficulties in isolating it from environmental sources do not provide unequivocal evidence about the role of water as a source of transmission of this microorganism.

Culture methods, immunological methods and molecular methods have been employed to detect *H. pylori* in the aquatic environment.

Several studies on the occurrence of *H. pylori* in sewage and drinking water samples have been carried out worldwide using molecular methods. In many of these surveys, it was not possible to isolate the bacterium using culture methods (Table 3).

Based on the findings that there is an association between water sources and the prevalence of *H. pylori* infection in Peruvian children[97], *H. pylori* DNA was detected in drinking water samples from different locations near Lima (Peru) in two different surveys using molecular methods. These results provided evidence of the presence of *H. pylori* DNA in drinking water in Peru and were consistent with conclusions from a previous epidemiological study of the same population[101,102]. In addition, other studies have highlighted the presence of *H. pylori* DNA in samples of tap water, well water[103], aquatic systems located in Mexico City[104], trucks for water transport and lake water[105], further supporting the hypothesis of the transmissibility of *H. pylori* through water.

Despite the high incidence of *H. pylori* DNA in water, only a few studies have reported bacteriological isolation of this microorganism. Bacteriological isolation of *H. pylori* occurred in the study of Lu *et al*[106], who analysed untreated municipal wastewater samples using a series of steps beginning with immunomagnetic separation and cell culture.

In a survey carried out in Iraq, out of 198 samples of treated municipal drinking water, 10 strains of *H. pylori* were isolated and identified. The low concentration of chlorine in the water samples and the ability to form biofilms in water pipes[107,108] were the reasons that *H. pylori* was isolated[109].

H. pylori was also isolated in tap water, dental unit water, and bottled mineral water in Iran. Out of 200 water samples collected in Iran, 5 cultures were positive. Two out of 50 tap water samples (4%), 2 out of 35 dental unit water samples (5.8%), and 1 out of 40 samples (2.5%) from water coolers in public places were found to be contaminated with *H. pylori*[110]. Ranjbar *et al*[111] examined 450 bottled mineral water samples and confirmed the presence of *H. pylori* in bottled mineral water.

Survival studies in water samples showed that *H. pylori* could be cultured from 48 h up to 20 d in autoclaved distilled water. An increase in survival occurs at low temperatures; in fact, high temperature causes loss of culturability[89,91,112-118]. Shahamat *et al*[118] used an autoradiographic method to detect the metabolic activity of *H. pylori* VBNC in water. Four *H. pylori* strains were studied using 72-h cultures in water and incubated with [3H] thymidine for 24-72 h. After being exposed to the Kodak NTB2 emulsion for 3-28 d, the organism was vital and culturable under these conditions for up to 48 h and, in some cases, 20 to 30 d (Table 3).

One factor in support of *H. pylori* infection being waterborne or related to poor health practices is the association, which some authors claim, of *H. pylori* with free-living amoebae (FLA), such as *Acanthamoeba*, *Naegleria*, *Vermamoeba* or *Balamuthia*, which are ubiquitous protozoa commonly found in water[119-122].

Several studies have also shown that *H. pylori* can be present as a biofilm on the pipes of the drinking water system with the ability to adhere to different hydraulic materials, such as copper and stainless steel[121,123,124]. As a result, *H. pylori* is likely to survive in an aquatic system within a biofilm rather than in the planktonic state[39,125].

H. pylori cells were able to survive for short periods in chlorinated drinking water in the VBNC form, which would allow them to reach final consumption points and, at the same time, enable them to be undetectable by culture methods[115].

Moreover, in biofilms, the resistance of *H. pylori* to chlorine increases significantly[120,126]. Therefore, it is possible that if the organism enters a distribution system, it may survive disinfection treatment within the biofilm matrix[115]. This characteristic may be based on the lack of isolation in some surveys of *H. pylori*. In fact, in water samples treated with suitable potabilization

Table 3 Studies evaluating the occurrence and survival of *Helicobacter pylori* in water

Year	Water and study type	Method	Observations	Ref.
1993	Survival of *H. pylori* in artificially contaminated sterile river water	Culture Autoradiography	Culture up to 48 h	Shahamat *et al*[118]
1996	Occurrence of *H. pylori* in 48 water samples: 30 from municipal water system, 14 from community taps, 4 from brick tanks or plastic barrels of different households	IMS and PCR	50% PCR positive samples	Hulten *et al*[101]
1997	Study on *H. pylori* resistance to chlorination	Culture	*H. pylori* were readily inactivated by free chlorine	Johnson *et al*[117]
1999	Occurrence of *H. pylori* in water from rivers and ponds	IMS and nested PCR	*H. pylori*-specific DNA was detected in samples	Sasaki *et al*[103]
1999	Occurrence of *H. pylori* in water from delivery truck and two lakes	Nested PCR and Southern blot hybridization	PCR positive samples from truck PCR positive samples from two lakes	McKeown *et al*[105]
2001	Occurrence of *H. pylori* in 10 seawater samples, 10 river water samples, 10 tap water samples, 6 well water samples	IMS, real-time PCR and nested PCR	2 PCR positive samples of well water	Horiuchi *et al*[127]
2001	Occurrence of *H. pylori* in 139 ground water samples	PCR and Southern blot hybridization	69% positive samples	Mazari-Hiriart *et al*[104]
2002	Occurrence of *H. pylori* in raw municipal wastewater	IMS, culture and PCR	23 out of 37 isolated strains were confirmed to be *H. pylori* 11 out of 23 strains of *H. pylori* demonstrated *vac*A gene heterogeneity	Lu *et al*[106]
2002	Study on the susceptibility of *H. pylori* to chlorine, monochloramine, and ozone compared to that of *Escherichia coli*	Culture	*H. pylori* was more resistant than *E. coli* to chlorine and ozone but not monochloramine	Baker *et al*[116]
2004	Occurrence of *H. pylori* in water and biofilms: 11 samples from domestic proprieties, 7 samples from educational properties and from hydrants, and samples from reservoirs and water meters of 3 water utilities	Culture, IMS and PCR	All cultures were negative 26% PCR positive sample with the highest frequency in biofilm	Watson *et al*[120]
2004	Occurrence of *H. pylori* in seawater	Nested-PCR	*H. pylori* DNA only detected in fractionated water samples containing zooplanktonic organisms	Cellini *et al*[130]
2005	Occurrence of *H. pylori* in seawater	Filtration (200 mm, filter), culture and PCR	*H. pylori* was only isolated from fractionated water samples containing large zooplanktonic organisms	Cellini *et al*[131]
2005	Occurrence of *H. pylori* in 36 seawater samples	Culture and PCR	30 positive samples	Carbone *et al*[132]
2006	Study on the ability of *H. pylori* to adhere on different water-exposed abiotic surfaces	Scanning electron microscope	*H. pylori* was able to adhere to all substrates tested	Azevedo *et al*[123]
2007	Study on the ability of *H. pylori* to adhere to stainless steel 304 in different environmental conditions	Epifluorescence microscopy	*H. pylori* was able to adhere to stainless steel 304	Azevedo *et al*[124]
2007	Study on the resistance of *H. pylori* to chlorination	Culture, FISH, PCR and RT-PCR	Culture until 5 min FISH viable cells until 3 h PCR samples positive after 24 h RT-PCR positive after 24 h	Moreno *et al*[115]
2007	Survival of *H. pylori* in spiked bottled mineral water (drinking water)	Culture epifluorescence microscopy and PCR	Culture until 5 d Cell viability until 14 d	Queralt *et al*[114]
2007	Survival of *H. pylori* in spiked chlorinated filtered water (drinking water)	Culture, FISH and PCR	Culture until 5 min FISH viable cells until 3 h PCR positive after 24 h RT-PCR after 24 h	Monero-Mesonero *et al*[115]
2009	Occurrence of *H. pylori* in 75 drinking and environmental water samples and 21 natural water biofilms samples	Real-time PCR	0% positive samples	Janzon *et al*[129]
2010	Occurrence of *H. pylori* in 198 drinking water samples	Culture	10 out of 469 isolated strains were confirmed *H. pylori*	Al-Sulami *et al*[109]
2011	Occurrence of *H. pylori* in 137 seawater samples	PCR	21% of the samples were positive for *H. pylori*	Twing *et al*[134]

2013	Occurrence of *H. pylori* in 50 tap water samples, 35 dental units' water samples, and 40 bottled mineral water samples	Culture and PCR	2 positive tap water samples 2 positive water from dental unit samples 1 positive water coolers sample	Bahrami *et al*[110]
2013	Occurrence of *H. pylori* in 31 seawater samples	Culture and PCR	4 positive samples	Holman *et al*[135]
2016	Occurrence of *H. pylori* in 450 bottled mineral water samples	Culture and PCR	8 positive samples	Ranjbar *et al*[111]
2018	Occurrence of *H. pylori* in 241 drinking water samples	PCR	49 positive samples	Boehnke *et al*[102]

H. pylori: Helicobacter pylori.

systems, the lack of isolation can be caused by the formation of the biofilm or by the conversion of *H. pylori* in the form of VBNC[127-129].

Some important clues have emerged from Italian research on the presence of bacteria in seawater. Work by Cellini *et al*[130,131] and subsequent investigations[132] suggest that a significant reservoir for the microorganism is seawater, in which *H. pylori* can occur both in a free-living form and in association with plankton. Plankton-related *H. pylori* cells were detected in both summer and winter months depending on the flowering of Copepods and Cladocerans. The authors supposed that zooplankton organisms represent a sort of protected niche for survival of the microorganism. The finding of *H. pylori* attached to planktonic organisms is particularly interesting for the role of the latter in the seafood chain and its subsequent potential role in the spread of *H. pylori* infection[130-132]. More generally, the presence of *H. pylori* in seawater could also be a health hazard for swimmers and others using those waters for work or pleasure[133].

Moreover, *H. pylori* DNA has been isolated in 21% of samples from freshwater, estuarine and beach sites in Delaware (United States)[134] and in seawater sampled from 31 locations in Georgia, Trinidad and Puerto Rico[135]. In both reports, no correlation between the occurrence of *H. pylori* and faecal indicator bacteria was found, suggesting that standard water quality tests are ineffective in predicting the presence of this pathogen in natural waters, confirming the potential risk for *H. pylori* presence in marine waters.

METHODS FOR THE DETECTION OF *H. PYLORI* IN FOODS AND WATER

The isolation of *H. pylori* from food samples, particularly when they present high loads of accompanying microflora, is demanding and time consuming because it requires the use of selective media with numerous antibiotics, microaerophilic conditions and long incubation periods (7 d)[55,71]. The detection of *H. pylori* in food samples and water by means of conventional microbiological techniques generally employed for clinical specimens, which are unable to detect the VBNC, may yield false negative results and thus underestimate the presence of the bacterium in food; furthermore, the presence of *H. pylori* in VBNC state in food and water represents a

potential microbiological risk for consumers, especially as a source of virulence factors[37,136,137].

Several solid and liquid culture media for the selective isolation of *H. pylori* from foods have been tested. The culture media most suitable for *H. pylori* growth often contain defibrinated horse or sheep blood acting as a reducing agent[29]. Furthermore, to achieve replication of *H. pylori* in broth culture, agitation is mandatory to provide good dispersion of gases throughout the liquid[31].

Brain heart infusion broth (BHIB) with growth supplement and selective agents has been evaluated[26,56]. BHIB with horse serum supplemented with porcine stomach mucin (0.3%), ferrous sulphate and sodium pyruvate (5%) or urea, along with the adjustment of the pH to 5.5 or 4.5, enhances the survival and possibly enables the growth of *H. pylori* in enrichment medium with fresh ground beef. In particular, pH 5.5 greatly enhances the growth and detectability of *H. pylori* in foods and should be considered an important factor for the detection of *H. pylori* in enrichment culture[56]. Stevenson *et al*[55] compared the growth of *H. pylori* in several liquid and solid media. None of the media tested presented an outstanding performance; only *H. pylori* special peptone agar offered the advantage of allowing the formation of the largest *H. pylori* colonies, and it was suitable for recovering *H. pylori* from environmental samples likely to be contaminated with large numbers of competing microorganisms[31,55].

Poms and Tatini[26] evaluated the efficacy for the *in vitro* isolation of *H. pylori* from foods of two solid media containing tryptic soy agar and Wilkins-Chalgren anaerobe agar supplemented with 5% defibrinated horse blood. The latter, to which several antibiotics (30 mg/L colistinmethanesulphonate, 100 mg/L cycloheximide, 30 mg/L nalidixic acid, 30 mg/L trimethoprim, and 10 mg/L vancomycin) were added, was found to be highly selective for the recovery of *H. pylori* from foods, but it lacked sufficient sensitivity to detect very low numbers of the bacterium.

Many authors have successfully used Wilkins-Chalgren anaerobe-agar or the broth developed by Poms *et al*[26] for the isolation of *H. pylori* in foods, both supplemented as described above[70,76,78-80].

However, there are still no standardized isolation protocols that are able to isolate the few *H. pylori* cells

present in samples rich in microbial flora such as food. Furthermore, the pathogen is able to enter a VBNC state that remains metabolically active but fails to develop into colonies when cultured on routine media[40]. Consequently, molecular assays have been conducted to detect *H. pylori* DNA in water and foodstuffs.

Immuno-separation (IMS) followed by PCR has been successfully used by several investigators[138-140]. The advantage of using this protocol is that it offers excellent specificity using the IMS able to concentrate the pathogen from foods, followed by high sensitivity of the molecular methods[29]. Nevertheless, it appears expensive, exacting and time consuming. Autoradiography and ATP bioluminescence have been successfully used for the detection of *H. pylori* from water, human stools and pure cultures but have never been tested on food[29,118]. In addition, the ATP bioluminescence assay does not allow for distinguishing among ATP from different cell sources when applied to a complex system such as a foodstuff[141].

A multiplex touchdown PCR (MT-PCR) method for the identification (16S rRNA gene) and genotyping (*vac*A- s1/m1, s1/m2, and s2/m2- and *cag*A genes) of *H. pylori* directly from artificially contaminated sheep milk was developed[142]. The characterization of the genes encoding virulence factors provides important information with respect to the sanitary assessment of food items because of the greater pathogenicity of certain *H. pylori* genotypes. Hence, for public health purposes, the evaluation of a food containing *H. pylori* will thus have to include the genotyping of isolates. This rapid, sensitive (15 cfu/mL) and specific molecular method presents the advantage of detecting and genotyping *H. pylori* from microbiologically complex foodstuffs in a single step[142]. A nested PCR approach has been employed for the detection of the *H. pylori glm*M gene from seawater and sheep, goat and cow milks[71,130,141]. The sensitivity of the nested PCR technique was 3 cfu/mL in all types of milk and 62 cfu/mL in seawater samples, and therefore, compared to the previously described MT-PCR, it was more sensitive for the detection of *H. pylori* from foods (3 cfu/mL *vs* 15 cfu/mL) with the same specificity[141]. Osman *et al*[73] employed nested PCR in 50 cow milk samples, and 22% were positive for the presence of the *H. pylori glm*M gene.

H. pylori, as with many other bacteria, is able to form biofilms within which it can survive due to the different protection mechanisms that the biofilm offers. Quantitative real-time PCR was developed for the detection of *H. pylori* in drinking water biofilms of different ages. The target gene was the *ure*A subunit of the *H. pylori urea* gene, which showed high specificity and sensitivity[143].

As is well known, the main limit of PCR assays is their inability to distinguish live organisms from dead organisms. PCR techniques can, however, be used to screen water and foodstuffs, thus making it necessary to use conventional isolation methods only on those samples that test positive by PCR.

In a study by Buck *et al*[58], mRNA of known virulence factor (*vac*A) was detected in VBNC *H. pylori* cells using RT-PCR. This method exploits the unstable nature of bacterial mRNA to infer pathogenic viability when *H. pylori* becomes non-cultivable[144]. The half-life of mRNA of *E. coli* and *Vibrio vulnificus* cells is approximately 3-8 min and less than 60 min, respectively[145,146]. Thus, mRNA can be an excellent indicator of viability when *H. pylori* occurs in the VBNC state. Moreover, detection of transcripts from the *vac*A virulence gene may deduce continued virulence activity of *H. pylori* when present in the VBNC state[58]. This molecular technique offers significant promise for the detection of microorganisms in water and foodstuff and is a valid alternative to culture methods.

The fluorescence in situ hybridization (FISH) assay with the rRNA-direct molecular method has been applied for the specific detection of *H. pylori* in river and wastewater samples[147] and in raw bulk tank bovine milk[67] and for the assessment of its survival in chlorinated drinking water[115,148]. The authors concluded that FISH was a rapid method for the direct detection and specific identification of viable bacteria in food[67].

CONCLUSION

Several studies report the presence and survival of *H. pylori* in foods and water, especially in milk and in ready-to-eat products, suggesting that they can be sources of infection for humans.

Although many of the findings reported in the literature are based on indirect evidence of *H. pylori* in food and water through molecular methods and there are only in a few cases on the bacteriological isolation of this microorganism, the possibility that food and water can be routes of transmission among others cannot be ruled out.

Most of the bacteriological isolations of the pathogen in foods and water have been obtained in work conducted in Iran; a possible explanation could be the greater prevalence of the disease in this geographical area than in other areas. Moreover, the discrepancy in the prevalence of *H. pylori* in the different surveys could be related to the type and number of samples tested, sampling method, experimental methodology and climate differences in the regions from which the samples were collected.

However, to confirm a definite foodborne and waterborne role of *H. pylori* transmission, more surveys are needed on the presence of *H. pylori* in other foods of animal origin, particularly in seafood, and on the survival ability of this microorganism in dry fermented sausages and dairy products. Further investigations on the possible role of humans and animals as reservoirs of the microorganism are also required to clarify the faecal-oral route of transmission and the method of food and water contamination. Finally, the development of molecular biology methods and, above all, bacteriological isolation methods of *H. pylori* from

water and food would add provide data that could confirm or deny the role of *H. pylori* as a foodborne and waterborne pathogen.

REFERENCES

1   **Marshall BJ**, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; **1**: 1311-1315 [PMID: 6145023 DOI: 10.1016/S0140-6736(84)91816-6]

2   **Marshall BJ.** History of the discovery of Campylobacter pylori. In: Blaser MJ. Campylobacter pylori in gastritis and peptic ulcer disease. New York: Igaku Shoin Publishers, 1989: 7-24

3   **Dunn BE**, Cohen H, Blaser MJ. Helicobacter pylori. *Clin Microbiol Rev* 1997; **10**: 720-741 [PMID: 9336670]

4   **Kao CY**, Sheu BS, Wu JJ. Helicobacter pylori infection: An overview of bacterial virulence factors and pathogenesis. *Biomed J* 2016; **39**: 14-23 [PMID: 27105595 DOI: 10.1016/j.bj.2015.06.002]

5   **Leja M**, Axon A, Brenner H. Epidemiology of Helicobacter pylori infection. *Helicobacter* 2016; **21** Suppl 1: 3-7 [PMID: 27531531 DOI: 10.1111/hel.12332]

6   **Kusters JG**, van Vliet AH, Kuipers EJ. Pathogenesis of Helicobacter pylori infection. *Clin Microbiol Rev* 2006; **19**: 449-490 [PMID: 16847081 DOI: 10.1128/CMR.00054-05]

7   **Hatakeyama M**, Brzozowski T. Pathogenesis of Helicobacter pylori infection. *Helicobacter* 2006; **11** Suppl 1: 14-20 [PMID: 16925606 DOI: 10.1111/j.1478-405X.2006.00424.x]

8   **Kuo CH**, Chen YH, Goh KL, Chang LL. Helicobacter pylori and Systemic Disease. *Gastroenterol Res Pract* 2014; **2014**: 358494 [PMID: 24772167 DOI: 10.1155/2014/358494]

9   **Torres J**, Pérez-Pérez G, Goodman KJ, Atherton JC, Gold BD, Harris PR, la Garza AM, Guarner J, Muñoz O. A comprehensive review of the natural history of Helicobacter pylori infection in children. *Arch Med Res* 2000; **31**: 431-469 [PMID: 11179581 DOI: 10.1016/S0188-4409(00)00099-0]

10   **Frenck RW Jr**, Clemens J. Helicobacter in the developing world. *Microbes Infect* 2003; **5**: 705-713 [PMID: 12814771 DOI: 10.1016/S1286-4579(03)00112-6]

11   **Olivares D**, Gisbert JP. Factors involved in the pathogenesis of Helicobacter pylori infection. *Rev Esp Enferm Dig* 2006; **98**: 374-386 [PMID: 16944998]

12   **Axon A**. How to influence health providers. *Helicobacter* 2007; **12** Suppl 2: 80-84 [PMID: 17991182 DOI: 10.1111/j.1523-5378.2007.00569.x]

13   **Singh M**, Prasad KN, Yachha SK, Saxena A, Krishnani N. Helicobacter pylori infection in children: prevalence, diagnosis and treatment outcome. *Trans R Soc Trop Med Hyg* 2006; **100**: 227-233 [PMID: 16260014 DOI: 10.1016/j.trstmh.2005.03.009]

14   **Ozaydin N**, Turkyilmaz SA, Cali S. Prevalence and risk factors of Helicobacter pylori in Turkey: a nationally-representative, cross-sectional, screening with the ¹³C-Urea breath test. *BMC Public Health* 2013; **13**: 1215 [PMID: 24359515 DOI: 10.1186/1471-2458-13-1215]

15   **Khedmat H**, Karbasi-Afshar R, Agah S, Taheri S. Helicobacter pylori Infection in the general population: A Middle Eastern perspective. *Caspian J Intern Med* 2013; **4**: 745-753 [PMID: 24294467]

16   **Perez-Perez GI**, Rothenbacher D, Brenner H. Epidemiology of Helicobacter pylori infection. *Helicobacter* 2004; **9** Suppl 1: 1-6 [PMID: 15347299 DOI: 10.1111/j.1083-4389.2004.00248.x]

17   **Breckan RK**, Paulssen EJ, Asfeldt AM, Kvamme JM, Straume B, Florholmen J. The All-Age Prevalence of Helicobacter pylori Infection and Potential Transmission Routes. A Population-Based Study. *Helicobacter* 2016; **21**: 586-595 [PMID: 27172105 DOI: 10.1111/hel.12316]

18   **Goodman KJ**, Correa P. The transmission of Helicobacter pylori. A critical review of the evidence. *Int J Epidemiol* 1995; **24**: 875-887 [PMID: 8557443 DOI: 10.1093/ije/24.5.875]

19   **Brown LM**. Helicobacter pylori: epidemiology and routes of transmission. *Epidemiol Rev* 2000; **22**: 283-297 [PMID: 11218379]

20   **Allaker RP**, Young KA, Hardie JM, Domizio P, Meadows NJ. Prevalence of helicobacter pylori at oral and gastrointestinal sites in children: evidence for possible oral-to-oral transmission. *J Med Microbiol* 2002; **51**: 312-317 [PMID: 11926736 DOI: 10.1099/0022-1317-51-4-312]

21   **Mitchell H**, Katelaris P. Epidemiology, clinical impacts and current clinical management of Helicobacter pylori infection. *Med J Aust* 2016; **204**: 376-380 [PMID: 27256648 DOI: 10.5694/mja16.00104]

22   **Singh V**, Trikha B, Vaiphei K, Nain CK, Thennarasu K, Singh K. Helicobacter pylori: evidence for spouse-to-spouse transmission. *J Gastroenterol Hepatol* 1999; **14**: 519-522 [PMID: 10385058 DOI: 10.1046/j.1440-1746.1999.01908.x]

23   **Schütze K**, Hentschel E, Dragosics B, Hirschl AM. Helicobacter pylori reinfection with identical organisms: transmission by the patients' spouses. *Gut* 1995; **36**: 831-833 [PMID: 7615268 DOI: 10.1136/gut.36.6.831]

24   **Kast RE**. Some fibrocystic breast change may be caused by sexually transmitted H. pylori during oral nipple contact: supporting literature and case report of resolution after gut H. pylori eradication treatment. *Med Hypotheses* 2007; **68**: 1041-1046 [PMID: 17113238 DOI: 10.1016/j.mehy.2006.09.050]

25   **Yee JKC**. Are the view of Helicobacter pylori colonized in the oral cavity an illusion? *Exp Mol Med* 2017; **49**: e397 [PMID: 29170474 DOI: 10.1038/emm.2017.225]

26   **Poms RE**, Tatini SR. Survival of Helicobacter pylori in ready-to-eat foods at 4 degrees C. *Int J Food Microbiol* 2001; **63**: 281-286 [PMID: 11246912 DOI: 10.1016/S0168-1605(00)00441-4]

27   **Wesley IV**. Helicobacter and Arcobacter: Potential human foodborne pathogens? *Trends Food Sci Technol* 1997; **8**: 293-299

28   **Meng J**, Doyle MP. Emerging issues in microbiological food safety. *Annu Rev Nutr* 1997; **17**: 255-275 [PMID: 9240928 DOI: 10.1146/annurev.nutr.17.1.255]

29   **Velázquez M**, Feirtag JM. Helicobacter pylori: characteristics, pathogenicity, detection methods and mode of transmission implicating foods and water. *Int J Food Microbiol* 1999; **53**: 95-104 [PMID: 10634701 DOI: 10.1016/S0168-1605(99)00160-9]

30   **van Duynhoven YT**, de Jonge R. Transmission of Helicobacter pylori: a role for food? *Bull World Health Organ* 2001; **79**: 455-460 [PMID: 11417041]

31   **Gomes BC**, De Martinis ECP. The significance of Helicobacter pylori in water, food and environmental samples. *Food Control* 2004; **15**: 397-403 [DOI: 10.1016/S0956-7135(03)00106-3]

32   **Solnick JV**, Schauer DB. Emergence of diverse Helicobacter species in the pathogenesis of gastric and enterohepatic diseases. *Clin Microbiol Rev* 2001; **14**: 59-97 [PMID: 11148003 DOI: 10.1128/CMR.14.1.59-97.2001]

33   **Romaniuk PJ**, Zoltowska B, Trust TJ, Lane DJ, Olsen GJ, Pace NR, Stahl DA. Campylobacter pylori, the spiral bacterium associated with human gastritis, is not a true Campylobacter sp. *J Bacteriol* 1987; **169**: 2137-2141 [PMID: 3571163 DOI: 10.1128/jb.169.5.2137-2141.1987]

34   **Goodwin CS**, Armstrong JA. Microbiological aspects of Helicobacter pylori (Campylobacter pylori). *Eur J Clin Microbiol Infect Dis* 1990; **9**: 1-13 [PMID: 2406141 DOI: 10.1007/BF01969526]

35   **Pinto D**, Santos MA, Chambel L. Thirty years of viable but nonculturable state research: unsolved molecular mechanisms. *Crit Rev Microbiol* 2015; **41**: 61-76 [PMID: 23848175 DOI: 10.3109/1040841X.2013.794127]

36   **Percival SL**, Yates MV, Williams DW, Chalmers RM, Gray NF. Helicobacter pylori . In: Microbiology of waterborne diseases. Microbiological aspects and risks. 2nd ed. London: Elsevier, 2014: 119-153. ISBN: 978-0-12-415846-7.

37   **Cellini L**. Helicobacter pylori: a chameleon-like approach to life. *World J Gastroenterol* 2014; **20**: 5575-5582 [PMID: 24914317 DOI: 10.3748/wjg.v20.i19.5575]

38   **Cellini L**, Robuffo I, Di Campli E, Di Bartolomeo S, Taraborelli T, Dainelli B. Recovery of Helicobacter pylori ATCC43504 from

a viable but not culturable state: regrowth or resuscitation? *APMIS* 1998; **106**: 571-579 [PMID: 9674895]

39   **Andersen LP**, Rasmussen L. Helicobacter pylori-coccoid forms and biofilm formation. *FEMS Immunol Med Microbiol* 2009; **56**: 112-115 [PMID: 19453756 DOI: 10.1111/j.1574-695X.2009.00556.x]

40   **Li L**, Mendis N, Trigui H, Oliver JD, Faucher SP. The importance of the viable but non-culturable state in human bacterial pathogens. *Front Microbiol* 2014; **5**: 258 [PMID: 24917854 DOI: 10.3389/fmicb.2014.00258]

41   **Lleò MM**, Bonato B, Tafi MC, Signoretto C, Boaretti M, Canepari P. Resuscitation rate in different enterococcal species in the viable but non-culturable state. *J Appl Microbiol* 2001; **91**: 1095-1102 [PMID: 11851818]

42   **Senoh M**, Ghosh-Banerjee J, Ramamurthy T, Colwell RR, Miyoshi S, Nair GB, Takeda Y. Conversion of viable but nonculturable enteric bacteria to culturable by co-culture with eukaryotic cells. *Microbiol Immunol* 2012; **56**: 342-345 [PMID: 22537150 DOI: 10.1111/j.1348-0421.2012.00440.x]

43   **Versalovic J**, Fox JG. Helicobacter. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH. Manual of Clinical Microbiology 7h edition 1999: 727-736.

44   **Lopes AI**, Vale FF, Oleastro M. Helicobacter pylori infection - recent developments in diagnosis. *World J Gastroenterol* 2014; **20**: 9299-9313 [PMID: 25071324 DOI: 10.3748/wjg.v20.i28.9299]

45   **Hachem CY**, Clarridge JE, Evans DG, Graham DY. Comparison of agar based media for primary isolation of Helicobacter pylori. *J Clin Pathol* 1995; **48**: 714-716 [PMID: 7560195 DOI: 10.1136/jcp.48.8.714]

46   **Henriksen TH**, Brorson O, Schöyen R, Thoresen T, Setegn D, Madebo T. Rapid growth of Helicobacter pylori. *Eur J Clin Microbiol Infect Dis* 1995; **14**: 1008-1011 [PMID: 8654438 DOI: 10.1007/BF01691385]

47   **Jiang X**, Doyle MP. Effect of environmental and substrate factors on survival and growth of Helicobacter pylori. *J Food Prot* 1998; **61**: 929-933 [PMID: 9713749 DOI: 10.4315/0362-028X-61.8.929]

48   **Roma-Giannikou E**, Karameris A, Balatsos B, Panayiotou J, Manika Z, Van-Vliet C, Rokkas T, Skandalis N, Kattamis C. Intrafamilial spread of Helicobacter pylori (H. pylori): A genetic analysis. European Helicobacter Study Group, Abstract from Athens Workshop 2002. XV International Workshop, September 11-14.

49   **Nurgalieva ZZ**, Malaty HM, Graham DY, Almuchambetova R, Machmudova A, Kapsultanova D, Osato MS, Hollinger FB, Zhangabylov A. Helicobacter pylori infection in Kazakhstan: effect of water source and household hygiene. *Am J Trop Med Hyg* 2002; **67**: 201-206 [PMID: 12389948 DOI: 10.4269/ajtmh.2002.67.201]

50   **Vaira D**, D'Anastasio C, Holton J, Dowsett J, Londei M, Salmon P, Gandolfi L. Is Campylobacter pylori a zoonosis? *Lancet* 1988; **2**: 1149 [PMID: 2903369]

51   **Husson MO**, Vincent P, Grabiaud MH, Furon D, Leclerc H. Anti-Helicobacter pylori IgG levels in abattoir workers. *Gastroenterol Clin Biol* 1991; **15**: 723-726 [PMID: 1816012]

52   **Beuchat LR**. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. *Microbes Infect* 2002; **4**: 413-423 [PMID: 11932192 DOI: 10.1016/S1286-4579(02)01555-1]

53   **Quaglia NC**, Dambrosio A, Normanno G, Parisi A, Firinu A, Lorusso V, Celano GV. Survival of Helicobacter pylori in artificially contaminated ultrahigh temperature and pasteurized milk. *Food Microbiol* 2007; **24**: 296-300 [PMID: 17188208 DOI: 10.1016/j.fm.2006.04.008]

54   **Fan XG**, Chua A, Li TG, Zeng QS. Survival of Helicobacter pylori in milk and tap water. *J Gastroenterol Hepatol* 1998; **13**: 1096-1098 [PMID: 9870794 DOI: 10.1111/j.1440-1746.1998.tb00582.x]

55   **Stevenson TH**, Bauer N, Lucia LM, Acuff GR. Attempts to isolate Helicobacter from cattle and survival of Helicobacter pylori in beef products. *J Food Prot* 2000; **63**: 174-178 [PMID: 10678420 DOI: 10.4315/0362-028X-63.2.174]

56   **Jiang X**, Doyle MP. Optimizing enrichment culture conditions for detecting Helicobacter pylori in foods. *J Food Prot* 2002; **65**: 1949-1954 [PMID: 12495015 DOI: 10.4315/0362-028X-65.12.1949]

57   **Gomes BC**, De Martinis ECP. Fate of Helicobacter pylori artificially inoculated in lettuce and carrot samples. *Braz J Microbiol* 2004; **35**: 145-150 [DOI: 10.1590/S1517-83822004000100024]

58   **Buck A**, Oliver JD. Survival of spinach-associated Helicobacter pylori in the viable but nonculturable state. *Food Control* 2010; **21**: 1150-1154 [DOI: 10.1016/j.foodcont.2010.01.010]

59   **Guner A**, Kav K, Tekinsen KK, Dogruer Y, Telli N. Survival of Helicobacter pylori in Turkish fermented sucuk and heat-treated sucuk during production. *J Food Prot* 2011; **74**: 2055-2061 [PMID: 22186045 DOI: 10.4315/0362-028X.JFP-11-019]

60   **Ng CG**, Loke MF, Goh KL, Vadivelu J, Ho B. Biofilm formation enhances Helicobacter pylori survivability in vegetables. *Food Microbiol* 2017; **62**: 68-76 [PMID: 27889168 DOI: 10.1016/j.fm.2016.10.010]

61   **Verma N**, Singh M. A disposable microbial based biosensor for quality control in milk. *Biosens Bioelectron* 2003; **18**: 1219-1224 [PMID: 12835039 DOI: 10.1016/S0956-5663(03)00085-X]

62   **West AP**, Millar MR, Tompkins DS. Effect of physical environment on survival of Helicobacter pylori. *J Clin Pathol* 1992; **45**: 228-231 [PMID: 1556231]

63   **Midolo PD**, Lambert JR, Hull R, Luo F, Grayson ML. In vitro inhibition of Helicobacter pylori NCTC 11637 by organic acids and lactic acid bacteria. *J Appl Bacteriol* 1995; **79**: 475-479 [PMID: 7592140 DOI: 10.1111/j.1365-2672.1995.tb03164.x]

64   **Dore MP**, Bilotta M, Vaira D, Manca A, Massarelli G, Leandro G, Atzei A, Pisanu G, Graham DY, Realdi G. High prevalence of Helicobacter pylori infection in shepherds. *Dig Dis Sci* 1999; **44**: 1161-1164 [PMID: 10389689 DOI: 10.1023/A:1026676223825]

65   **Dore MP**, Sepulveda AR, El-Zimaity H, Yamaoka Y, Osato MS, Mototsugu K, Nieddu AM, Realdi G, Graham DY. Isolation of Helicobacter pylori from sheep-implications for transmission to humans. *Am J Gastroenterol* 2001; **96**: 1396-1401 [PMID: 11374673 DOI: 10.1111/j.1572-0241.2001.03772.x]

66   **Fujimura S**, Kawamura T, Kato S, Tateno H, Watanabe A. Detection of Helicobacter pylori in cow's milk. *Lett Appl Microbiol* 2002; **35**: 504-507 [PMID: 12460433 DOI: 10.1046/j.1472-765X.2002.01229.x]

67   **Angelidis AS**, Tirodimos I, Bobos M, Kalamaki MS, Papageorgiou DK, Arvanitidou M. Detection of Helicobacter pylori in raw bovine milk by fluorescence in situ hybridization (FISH). *Int J Food Microbiol* 2011; **151**: 252-256 [PMID: 21974980 DOI: 10.1016/j.ijfoodmicro.2011.09.007]

68   **Rahimi E**, Kheirabadi EK. Detection of Helicobacter pylori in bovine, buffalo, camel, ovine, and caprine milk in Iran. *Foodborne Pathog Dis* 2012; **9**: 453-456 [PMID: 22458716 DOI: 10.1089/fpd.2011.1060]

69   **Mousavi S**, Dehkordi FS, Rahimi E. Virulence factors and antibiotic resistance of Helicobacter pylori isolated from raw milk and unpasteurized dairy products in Iran. *J Venom Anim Toxins Incl Trop Dis* 2014; **20**: 51 [PMID: 25873940 DOI: 10.1186/1678-9199-20-51]

70   **Saeidi E**, Sheikhshahrokh A. vacA Genotype Status of Helicobacter pylori Isolated from Foods with Animal Origin. *Biomed Res Int* 2016; **2016**: 8701067 [PMID: 27088092 DOI: 10.1155/2016/8701067]

71   **Quaglia NC**, Dambrosio A, Normanno G, Parisi A, Patrono R, Ranieri G, Rella A, Celano GV. High occurrence of Helicobacter pylori in raw goat, sheep and cow milk inferred by glmM gene: a risk of food-borne infection? *Int J Food Microbiol* 2008; **124**: 43-47 [PMID: 18372071 DOI: 10.1016/j.ijfoodmicro.2008.02.011]

72   **Talaei R**, Souod N, Momtaz H, Dabiri H. Milk of livestock as a possible transmission route of Helicobacter pylori infection. *Gastroenterol Hepatol Bed Bench* 2015; **8**: S30-S36 [PMID: 26171135 DOI: 10.22037/ghfbb.v8iSupplement.764]

73   **Osman EY**, El-Eragi AM, Musa AM, El-Magboul SB, A/Rahman MB, Abdo AE. Detection of Helicobacter pylori glmM gene in bovine milk using Nested polymerase chain reaction.

74 **Turutoglu H**, Mudul S. Investigation of Helicobacter pylori in raw sheep milk samples. *J Vet Med B Infect Dis Vet Public Health* 2002; **49**: 308-309 [PMID: 12241034 DOI: 10.1046/j.1439-0450.2002.00557.x]

75 **Bianchini V**, Recordati C, Borella L, Gualdi V, Scanziani E, Selvatico E, Luini M. Helicobacteraceae in Bulk Tank Milk of Dairy Herds from Northern Italy. *Biomed Res Int* 2015; **2015**: 639521 [PMID: 26090429 DOI: 10.1155/2015/639521]

76 **Gilani A**, Razavilar V, Rokni N, Rahimi E. *VacA* and *cagA* genotypes status and antimicrobial resistance properties of *Helicobacter pylori* strains isolated from meat products in Isfahan province, Iran. *Iran J Vet Res* 2017; **18**: 97-102 [PMID: 28775748 DOI: 10.22099/ijvr.2017.4088]

77 **Meng X**, Zhang H, Law J, Tsang R, Tsang T. Detection of Helicobacter pylori from food sources by a novel multiplex PCR assay. *J Food Saf* 2008; **28**: 609-619 [DOI: 10.1111/j.1745-4565.2008.00135.x]

78 **Hemmatinezhad B**, Momtaz H, Rahimi E. VacA, cagA, iceA and oipA genotypes status and antimicrobial resistance properties of Helicobacter pylori isolated from various types of ready to eat foods. *Ann Clin Microbiol Antimicrob* 2016; **15**: 2 [PMID: 26792758 DOI: 10.1186/s12941-015-0115-z]

79 **Ghorbani F**, Gheisari E, Dehkordi FS. Genotyping of vacA alleles of Helicobacter pylori strains recovered from some Iranian food items. *Trop J Pharm Res* 2016; **15**: 1631-1636 [DOI: 10.4314/tjpr.v15i8.5]

80 **Yahaghi E**, Khamesipour F, Mashayekhi F, Safarpoor Dehkordi F, Sakhaei MH, Masoudimanesh M, Khameneie MK. Helicobacter pylori in vegetables and salads: genotyping and antimicrobial resistance properties. *Biomed Res Int* 2014; **2014**: 757941 [PMID: 25184146 DOI: 10.1155/2014/757941]

81 **Atapoor S**, Safarpoor Dehkordi F, Rahimi E. Detection of Helicobacter pylori in Various Types of Vegetables and Salads. *Jundishapur J Microbiol* 2014; **7**: e10013 [PMID: 25147709 DOI: 10.5812/jjm.10013]

82 **Thomson MA**, Storey P, Greer R, Cleghorn GJ. Canine-human transmission of Gastrospirillum hominis. *Lancet* 1994; **343**: 1605-1607 [PMID: 7911923 DOI: 10.1016/S0140-6736(94)93060-0]

83 **Dimola S**, Caruso ML. Helicobacter pylori in animals affecting the human habitat through the food chain. *Anticancer Res* 1999; **19**: 3889-3894 [PMID: 10628327]

84 **Quaglia NC**, Dambrosio A, Normanno G, Alberti F, Rella A, Tamborrino C, Celano GV. Detection of Helicobacter pylori in gastric mucosa of sheep: preliminary results. *Rivista dell' Associazione Italiana Veterinari Igienisti* 2009; **3**: 45-48 [DOI: 10.4081/ijfs.2009.1420]

85 **Papiez D**, Konturek PC, Bielanski W, Plonka M, Dobrzanska M, Kaminska A, Szczyrk U, Bochenek A, Wierzchos E. Prevalence of Helicobacter pylori infection in Polish shepherds and their families. *Dig Liver Dis* 2003; **35**: 10-15 [PMID: 12725601 DOI: 10.1016/S1590-8658(02)00004-X]

86 **Mladenova-Hristova I**, Grekova O, Patel A. Zoonotic potential of Helicobacter spp. *J Microbiol Immunol Infect* 2017; **50**: 265-269 [PMID: 28655571 DOI: 10.1016/j.jmii.2016.11.003]

87 World Health Organization (WHO) and the United Nations Children's Fund (UNICEF). Progress on drinking water, sanitation and hygiene: 2017 update and Sustainable Development Goal baselines. The Joint Monitoring Programme (JMP) report. 2018. Available from: URL: https://www.unicef.org/publications/index_96611.html

88 **Lambert JR**, Lin SK, Aranda-Michel J. Helicobacter pylori. *Scand J Gastroenterol Suppl* 1995; **208**: 33-46 [PMID: 7777803 DOI: 10.3109/00365529509107760]

89 **Vu C**, Ng YY. Prevalence of Helicobacter pylori in peptic ulcer disease in a Singapore hospital. *Singapore Med J* 2000; **41**: 478-481 [PMID: 11281437]

90 **Adams BL**, Bates TC, Oliver JD. Survival of Helicobacter pylori in a natural freshwater environment. *Appl Environ Microbiol* 2003;

69: 7462-7466 [PMID: 14660399 DOI: 10.1128/AEM.69.12.7462-7466.2003]

91 **Aziz RK**, Khalifa MM, Sharaf RR. Contaminated water as a source of Helicobacter pylori infection: A review. *J Adv Res* 2015; **6**: 539-547 [PMID: 26199743 DOI: 10.1016/j.jare.2013.07.007]

92 **Bellack NR**, Koehoorn MW, MacNab YC, Morshed MG. A conceptual model of water's role as a reservoir in Helicobacter pylori transmission: a review of the evidence. *Epidemiol Infect* 2006; **134**: 439-449 [PMID: 16512966 DOI: 10.1017/S0950268806006005]

93 **Lee YY**, Ismail AW, Mustaffa N, Musa KI, Majid NA, Choo KE, Mahendra Raj S, Derakhshan MH, Malaty HM, Graham DY. Sociocultural and dietary practices among Malay subjects in the north-eastern region of Peninsular Malaysia: a region of low prevalence of Helicobacter pylori infection. *Helicobacter* 2012; **17**: 54-61 [PMID: 22221617 DOI: 10.1111/j.1523-5378.2011.00917.x]

94 **Porras C**, Nodora J, Sexton R, Ferreccio C, Jimenez S, Dominguez RL, Cook P, Anderson G, Morgan DR, Baker LH, Greenberg ER, Herrero R. Epidemiology of Helicobacter pylori infection in six Latin American countries (SWOG Trial S0701). *Cancer Causes Control* 2013; **24**: 209-215 [PMID: 23263777 DOI: 10.1007/s10552-012-0117-5]

95 **Syam AF**, Miftahussurur M, Makmun D, Nusi IA, Zain LH, Zulkhairi, Akil F, Uswan WB, Simanjuntak D, Uchida T, Adi P, Utari AP, Rezkitha YA, Subsomwong P, Nasronudin, Suzuki R, Yamaoka Y. Risk Factors and Prevalence of Helicobacter pylori in Five Largest Islands of Indonesia: A Preliminary Study. *PLoS One* 2015; **10**: e0140186 [PMID: 26599790 DOI: 10.1371/journal.pone.0140186]

96 **Awuku YA**, Simpong DL, Alhassan IK, Tuoyire DA, Afaa T, Adu P. Prevalence of helicobacter pylori infection among children living in a rural setting in Sub-Saharan Africa. *BMC Public Health* 2017; **17**: 360 [PMID: 28438158 DOI: 10.1186/s12889-017-4274-z]

97 **Klein PD**, Graham DY, Gaillour A, Opekun AR, Smith EO. Water source as risk factor for Helicobacter pylori infection in Peruvian children. Gastrointestinal Physiology Working Group. *Lancet* 1991; **337**: 1503-1506 [PMID: 1675369 DOI: 10.1016/0140-6736(91)93196-G]

98 **Souto FJ**, Fontes CJ, Rocha GA, de Oliveira AM,Mendes EM, Queiroz DM. Prevalence of Helicobacter pylori Infection in a Rural Area of the State of Mato Grosso, Brazil. *Memorias do Instituto Oswaldo Cruz* 1998; **93**: 171-174 [DOI: 10.1590/S0074-02761998000200006]

99 **Herbarth O**, Krumbiegel P, Fritz GJ, Richter M, Schlink U, Müller DM, Richter T. Helicobacter pylori prevalences and risk factors among school beginners in a German urban center and its rural county. *Environ Health Perspect* 2001; **109**: 573-577 [PMID: 11445510]

100 **Fujimura S**, Kato S, Kawamura T. Helicobacter pylori in Japanese river water and its prevalence in Japanese children. *Lett Appl Microbiol* 2004; **38**: 517-521 [PMID: 15130149 DOI: 10.1111/j.1472-765X.2004.01529.x]

101 **Hulten K**, Han SW, Enroth H, Klein PD, Opekun AR, Gilman RH, Evans DG, Engstrand L, Graham DY, El-Zaatari FA. Helicobacter pylori in the drinking water in Peru. *Gastroenterology* 1996; **110**: 1031-1035 [PMID: 8612990 DOI: 10.1053/gast.1996.v110.pm8612990]

102 **Boehnke KF**, Brewster RK, Sánchez BN, Valdivieso M, Bussalleu A, Guevara M, Saenz CG, Alva SO, Gil E, Xi C. An assessment of drinking water contamination with Helicobacter pylori in Lima, Peru. *Helicobacter* 2018; **23**: e12462 [PMID: 29316052]

103 **Sasaki K**, Tajiri Y, Sata M, Fujii Y, Matsubara F, Zhao M, Shimizu S, Toyonaga A, Tanikawa K. Helicobacter pylori in the natural environment. *Scand J Infect Dis* 1999; **31**: 275-279 [PMID: 10482057]

104 **Mazari-Hiriart M**, López-Vidal Y, Calva JJ. Helicobacter pylori in water systems for human use in Mexico City. *Water Sci Technol* 2001; **43**: 93-98 [PMID: 11464777]

105 **McKeown I**, Orr P, Macdonald S, Kabani A, Brown R, Coghlan G, Dawood M, Embil J, Sargent M, Smart G, Bernstein CN.

Helicobacter pylori in the Canadian arctic: seroprevalence and detection in community water samples. *Am J Gastroenterol* 1999; **94**: 1823-1829 [PMID: 10406242 DOI: 10.1111/j.1572-0241.1999.01212.x]

106   **Lu Y**, Redlinger TE, Avitia R, Galindo A, Goodman K. Isolation and genotyping of Helicobacter pylori from untreated municipal wastewater. *Appl Environ Microbiol* 2002; **68**: 1436-1439 [PMID: 11872498 DOI: 10.1128/AEM.68.3.1436-1439.2002]

107   **Al-Taee MR**. Assessment of water quality due to microbial growth in drinking water distribution systems in Basrah city. *Marina Mesopotamica* 2001; **16**: 37-46

108   **Momba MNB**, Makala N. Comparing the effect of various pipe materials on biofilm formation in chlorinated and combined chlorine-chloraminated water systems. *Water SA* 2003; **30**: 175-182 [DOI: 10.4314/wsa.v30i2.5061]

109   **Al-Sulami AA**, Al-Taee AM, Juma'a MG. Isolation and identification of Helicobacter pylori from drinking water in Basra governorate, Iraq. *East Mediterr Health J* 2010; **16**: 920-925 [PMID: 21218716]

110   **Bahrami AR**, Rahimi E, Ghasemian Safaei H. Detection of Helicobacter pylori in city water, dental units' water, and bottled mineral water in Isfahan, Iran. *ScientificWorldJournal* 2013; **2013**: 280510 [PMID: 23606812 DOI: 10.1155/2013/280510]

111   **Ranjbar R**, Khamesipour F, Jonaidi-Jafari N, Rahimi E. Helicobacter pylori in bottled mineral water: genotyping and antimicrobial resistance properties. *BMC Microbiol* 2016; **16**: 40 [PMID: 26970903 DOI: 10.1186/s12866-016-0647-1]

112   **Vale FF**, Vítor JM. Transmission pathway of Helicobacter pylori: does food play a role in rural and urban areas? *Int J Food Microbiol* 2010; **138**: 1-12 [PMID: 20122750 DOI: 10.1016/j.ijfoodmicro.2010.01.016]

113   **Azevedo NF**, Almeida C, Cerqueira L, Dias S, Keevil CW, Vieira MJ. Coccoid form of Helicobacter pylori as a morphological manifestation of cell adaptation to the environment. *Appl Environ Microbiol* 2007; **73**: 3423-3427 [PMID: 17400788 DOI: 10.1128/AEM.00047-07]

114   **Queralt N**, Araujo R. Analysis of the survival of H. pylori within a laboratory-based aquatic model system using molecular and classical techniques. *Microb Ecol* 2007; **54**: 771-777 [PMID: 17401696 DOI: 10.1007/s00248-007-9242-1]

115   **Moreno Y**, Piqueres P, Alonso JL, Jiménez A, González A, Ferrús MA. Survival and viability of Helicobacter pylori after inoculation into chlorinated drinking water. *Water Res* 2007; **41**: 3490-3496 [PMID: 17585990 DOI: 10.1016/j.watres.2007.05.020]

116   **Baker KH**, Hegarty JP, Redmond B, Reed NA, Herson DS. Effect of oxidizing disinfectants (chlorine, monochloramine, and ozone) on Helicobacter pylori. *Appl Environ Microbiol* 2002; **68**: 981-984 [PMID: 11823249 DOI: 10.1128/AEM.68.2.981-984.2002]

117   **Johnson CH**, Rice EW, Reasoner DJ. Inactivation of Helicobacter pylori by chlorination. *Appl Environ Microbiol* 1997; **63**: 4969-4970 [PMID: 9406419]

118   **Shahamat M**, Mai U, Paszko-Kolva C, Kessel M, Colwell RR. Use of autoradiography to assess viability of Helicobacter pylori in water. *Appl Environ Microbiol* 1993; **59**: 1231-1235 [PMID: 8489232]

119   **Winiecka-Krusnell J**, Wreiber K, von Euler A, Engstrand L, Linder E. Free-living amoebae promote growth and survival of Helicobacter pylori. *Scand J Infect Dis* 2002; **34**: 253-256 [PMID: 12064686 DOI: 10.1080/00365540110080052]

120   **Watson CL**, Owen RJ, Said B, Lai S, Lee JV, Surman-Lee S, Nichols G. Detection of Helicobacter pylori by PCR but not culture in water and biofilm samples from drinking water distribution systems in England. *J Appl Microbiol* 2004; **97**: 690-698 [PMID: 15357718 DOI: 10.1111/j.1365-2672.2004.02360.x]

121   **Percival SL**, Thomas JG. Transmission of Helicobacter pylori and the role of water and biofilms. *J Water Health* 2009; **7**: 469-477 [PMID: 19491497 DOI: 10.2166/wh.2009.070]

122   **Moreno-Mesonero L**, Moreno Y, Alonso JL, Ferrús MA. Detection of viable Helicobacter pylori inside free-living amoebae in wastewater and drinking water samples from Eastern Spain.

123   **Azevedo NF**, Pacheco AP, Keevil CW, Vieira MJ. Adhesion of water stressed Helicobacter pylori to abiotic surfaces. *J Appl Microbiol* 2006; **101**: 718-724 [PMID: 16907822 DOI: 10.1111/j.1365-2672.2006.03029.x]

124   **Azevedo NF**, Pinto AR, Reis NM, Vieira MJ, Keevil CW. Shear stress, temperature, and inoculation concentration influence the adhesion of water-stressed Helicobacter pylori to stainless steel 304 and polypropylene. *Appl Environ Microbiol* 2006; **72**: 2936-2941 [PMID: 16598000 DOI: 10.1128/AEM.72.4.2936-2941.2006]

125   **Costerton JW**, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* 1999; **284**: 1318-1322 [PMID: 10334980 DOI: 10.1126/science.284.5418.1318]

126   **Yu FP**, McFeters GA. Physiological responses of bacteria in biofilms to disinfection. *Appl Environ Microbiol* 1994; **60**: 2462-2466 [PMID: 8074525]

127   **Horiuchi T**, Ohkusa T, Watanabe M, Kobayashi D, Miwa H, Eishi Y. Helicobacter pylori DNA in drinking water in Japan. *Microbiol Immunol* 2001; **45**: 515-519 [PMID: 11529557 DOI: 10.1111/j.1348-0421.2001.tb02652.x]

128   **Böckelmann U**, Dörries HH, Ayuso-Gabella MN, Salgot de Marçay M, Tandoi V, Levantesi C, Masciopinto C, Van Houtte E, Szewzyk U, Wintgens T, Grohmann E. Quantitative PCR monitoring of antibiotic resistance genes and bacterial pathogens in three European artificial groundwater recharge systems. *Appl Environ Microbiol* 2009; **75**: 154-163 [PMID: 19011075 DOI: 10.1128/AEM.01649-08]

129   **Janzon A**, Sjöling A, Lothigius A, Ahmed D, Qadri F, Svennerholm AM. Failure to detect Helicobacter pylori DNA in drinking and environmental water in Dhaka, Bangladesh, using highly sensitive real-time PCR assays. *Appl Environ Microbiol* 2009; **75**: 3039-3044 [PMID: 19304824]

130   **Cellini L**, Del Vecchio A, Di Candia M, Di Campli E, Favaro M, Donelli G. Detection of free and plankton-associated Helicobacter pylori in seawater. *J Appl Microbiol* 2004; **97**: 285-292 [PMID: 15239694 DOI: 10.1111/j.1365-2672.2004.02307.x]

131   **Cellini L**, Grand R, Prenna M, Pasquantonio M, Pane L. Detection of Helicobacter pylori associated with zooplankton. *Aquat Microb Ecol* 2005; **40**: 115-120 [DOI: 10.3354/ame040115]

132   **Carbone M**, Maugeri TL, Gugliandolo C, La Camera E, Biondo C, Fera MT. Occurrence of Helicobacter pylori DNA in the coastal environment of southern Italy (Straits of Messina). *J Appl Microbiol* 2005; **98**: 768-774 [PMID: 15715881 DOI: 10.1111/j.1365-2672.2004.02517.x]

133   **Dixon B**. Helicobacter from seas? ASM news 2005; **71**: 4-5

134   **Twing KI**, Kirchman DL, Campbell BJ. Temporal study of Helicobacter pylori presence in coastal freshwater, estuary and marine waters. *Water Res* 2011; **45**: 1897-1905 [PMID: 21193216 DOI: 10.1016/j.watres.2010.12.013]

135   **Holman CB**, Bachoon DS, Otero E, Ramsubhag A. Detection of Helicobacter pylori in the coastal waters of Georgia, Puerto Rico and Trinidad. *Mar Pollut Bull* 2014; **79**: 354-358 [PMID: 24332757 DOI: 10.1016/j.marpolbul.2013.11.021]

136   **Bode G**, Mauch F, Malfertheiner P. The coccoid forms of Helicobacter pylori. Criteria for their viability. *Epidemiol Infect* 1993; **111**: 483-490 [PMID: 8270008]

137   **Cao J**, Li ZQ, Borch K, Petersson F, Mårdh S. Detection of spiral and coccoid forms of Helicobacter pylori using a murine monoclonal antibody. *Clin Chim Acta* 1997; **267**: 183-196 [PMID: 9469252 DOI: 10.1016/S0009-8981(97)00134-4]

138   **Cohen AE**. A PCR method for the detection of Helicobacter pylori in food products: US Food and Drug Administration (FDA) Study. Proceedings of the 16th Food Microbiology Symposium "Current concepts in foodborne pathogens and rapid and automated methods in food microbiology"; 1996 University of Wisconsin, River Falls

139   **Enroth H**, Engstrand L. Immunomagnetic separation and PCR for detection of Helicobacter pylori in water and stool specimens. *J Clin Microbiol* 1995; **33**: 2162-2165 [PMID: 7559969]

140   **Hultén K**, Enroth H, Nyström T, Engstrand L. Presence

Environ Microbiol 2017; **19**: 4103-4112 [PMID: 28707344 DOI: 10.1111/1462-2920.13856]

of Helicobacter species DNA in Swedish water. *J Appl Microbiol* 1998; **85**: 282-286 [PMID: 9750301 DOI: 10.1046/j.1365-2672.1998.00500.x]

141 **Quaglia NC**, Dambrosio A, Normanno G, Celano GV. Evaluation of a Nested-PCR assay based on the phosphoglucosamine mutase gene (glmM) for the detection of Helicobacter pylori from raw milk. *Food Control* 2009; **20**: 119-123

142 **Quaglia NC**, Normanno G, Dambrosio A, Celano GV, Parisi A, Firinu A, Buonavoglia C. Multiplex-touchdown PCR assay for the detection and genotyping of Helicobacter pylori from artificially contaminated sheep milk. *J Food Prot* 2005; **68**: 2136-2139 [PMID: 16245719 DOI: 10.4315/0362-028X-68.10.2136]

143 **Linke S**, Lenz J, Gemein S, Exner M, Gebel J. Detection of Helicobacter pylori in biofilms by real-time PCR. *Int J Hyg Environ Health* 2010; **213**: 176-182 [PMID: 20427237 DOI: 10.1016/j.ijheh.2010.03.006]

144 **Lahtinen SJ**, Ahokoski H, Reinikainen JP, Gueimonde M, Nurmi J, Ouwehand AC, Salminen SJ. Degradation of 16S rRNA and attributes of viability of viable but nonculturable probiotic bacteria.

Lett Appl Microbiol 2008; **46**: 693-698 [PMID: 18444975 DOI: 10.1111/j.1472-765X.2008.02374.x]

145 **Sheridan GE**, Masters CI, Shallcross JA, MacKey BM. Detection of mRNA by reverse transcription-PCR as an indicator of viability in Escherichia coli cells. *Appl Environ Microbiol* 1998; **64**: 1313-1318 [PMID: 9546166]

146 **Smith B**, Oliver JD. In situ and in vitro gene expression by Vibrio vulnificus during entry into, persistence within, and resuscitation from the viable but nonculturable state. *Appl Environ Microbiol* 2006; **72**: 1445-1451 [PMID: 16461698 DOI: 10.1128/AEM.72.2.1445-1451.2006]

147 **Moreno Y**, Ferrús MA, Alonso JL, Jiménez A, Hernández J. Use of fluorescent in situ hybridization to evidence the presence of Helicobacter pylori in water. *Water Res* 2003; **37**: 2251-2256 [PMID: 12691913 DOI: 10.1016/S0043-1354(02)00624-3]

148 **Piqueres P**, Moreno Y, Alonso JL, Ferrús MA. A combination of direct viable count and fluorescent in situ hybridization for estimating Helicobacter pylori cell viability. *Res Microbiol* 2006; **157**: 345-349 [PMID: 16380234 DOI: 10.1016/j.resmic.2005.09.003]

Hepatitis B virus infection: Defective surface antigen expression and pathogenesis

Chun-Chen Wu, Ying-Shan Chen, Liang Cao, Xin-Wen Chen, Meng-Ji Lu

Chun-Chen Wu, Ying-Shan Chen, Liang Cao, Xin-Wen Chen, State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, Hubei Province, China

Liang Cao, Department of Microbiology and Immunology, Feinberg School of Medicine Northwestern University, Chicago, IL 60611, United States

Meng-Ji Lu, Institute of Virology, University Hospital of Essen, Essen 45122, Germany

ORCID number: Chun-Chen Wu (0000-0002-6888-213X); Ying-Shan Chen (0000-0002-2276-4496); Liang Cao (0000-0001-5974-9815); Xin-Wen Chen (0000-0002-4052-8155); Meng-Ji Lu (0000-0003-4287-9941).

Abstract

Hepatitis B virus (HBV) infection is a global public health concern. HBV causes chronic infection in patients and can lead to liver cirrhosis, hepatocellular carcinoma, and other severe liver diseases. Thus, understanding HBV-related pathogenesis is of particular importance for prevention and clinical intervention. HBV surface antigens are indispensable for HBV virion formation and are useful viral markers for diagnosis and clinical assessment. During chronic HBV infection, HBV genomes may acquire and accumulate mutations and deletions, leading to the expression of defective HBV surface antigens. These defective HBV surface antigens have been found to play important roles in the progression of HBV-associated liver diseases. In this review, we focus our discussion on the nature of defective HBV surface antigen mutations and their contribution to the pathogenesis of fulminant hepatitis B. The relationship between defective surface antigens and occult HBV infection are also discussed.

Key words: Hepatitis B surface protein; Defective surface antigen mutants; Endoplasmic reticulum stress; Fulminant hepatitis B; Occult hepatitis B virus infection; Pathogenesis

Core tip: Defective surface antigen mutation is a type of mutation with great clinical relevance. Many previous publications have explored the association of defective surface antigen mutation with the development of hepatitis B virus (HBV)-associated hepatocellular carcinoma. However, there are no reviews available that elaborate on the relationship between defective surface antigen mutation and HBV-associated fulminant hepatitis (FH), as well as occult hepatitis B virus infection (OBI). This review will focus on these two aspects to discuss the nature of defective HBV surface antigen mutations and their contribution to the pathogenesis of FH. The relationship between defective surface antigens and OBI are also discussed.

INTRODUCTION

Hepatitis B virus (HBV) is an important human pathogen that has caused chronic infections worldwide[1]. Recent data obtained from a modeling study has shown that the global prevalence of hepatitis B surface antigen (HBsAg) was 3.9% in 2016, corresponding to an estimated 290 million infections worldwide[2]. HBV mainly infects hepatocytes and causes a wide spectrum of clinical manifestations, ranging from an asymptomatic carrier state to acute or chronic hepatitis, with progression to liver cirrhosis, hepatocellular carcinoma (HCC), and other severe liver diseases[3,4]. Currently, interferon-α and nucleotide analogs are used to treat chronic HBV (CHB) infections; however, the outcome is far from satisfactory[5,6]. Prophylaxis using the current HBV vaccines has no impact on existing infections. Therapeutic vaccines of chronic HBV infection are under investigation, but further development is still required[7]. Therefore, understanding the molecular pathogenesis of HBV infection will provide opportunities for the development of better therapies and vaccines.

HBV belongs to the family *Hepadnaviridae* and is a small, enveloped virus with a partially double-stranded DNA genome approximately 3.2 kb in size[8]. During the life cycle of HBV, pre-genomic RNA (pgRNA) is transcribed from covalently closed circular DNA (cccDNA) and serves as the template for HBV DNA replication through a viral polymerase-mediated reverse transcription[9,10]. Because viral polymerase lacks a proof-reading function, the HBV genome evolves with an estimated rate of nucleotide substitutions of 1×10^{-3} to 1×10^{-6} per replication cycle, according to various

investigators[11]. Although HBV genome replication involves a step of reverse transcription, which is similar to retroviral replication, the complex HBV genome structure with overlapping open reading frames and regulatory sequences apparently limits the spectrum and rate of mutations[3,12]. Nevertheless, this unique replication strategy leads to the great diversity of HBV genomes, thus resulting in the occurrence of various genotypes, subtypes, mutants, recombinants, and even viral quasi-species in the context of long-term HBV evolution[13,14]. Several reports have suggested that the emergence of HBV variants plays important roles in the progression of HBV-associated liver diseases[11,15-18]. Defective surface antigen mutation is a type of mutation with great clinical relevance[11,15,19]. In this review, we report the current information on HBV surface antigen mutations. Further, we focus our discussion on the contribution of defective surface antigen mutations on the pathogenesis of HBV-associated liver diseases.

BIOLOGY OF HBV SURFACE ANTIGEN

Three viral envelope/surface proteins - large surface antigens (LHBs), middle surface antigens (MHBs), and small surface antigens (SHBs) - are expressed from a single open reading frame (S-ORF)[20,21], but they are translated from two different mRNAs. LHBs are encoded by the 2.4 kb subgenomic RNA, and MHBs and SHBs are encoded by the 2.1 kb subgenomic RNA[3]. Subgenomic RNAs of 2.4 kb and 2.1 kb are driven by preS1 and preS2/S promoters, respectively, allowing variable regulation of protein expression[3]. The preS1 promoter is situated within the upstream region of the S-ORF, whereas the preS2 promoter corresponds to the preS1 domain[21]. Therefore, the transcription of the 2.1 kb subgenomic RNA is also regulated by the preS1 domain[11] (Figure 1).

The three surface proteins share the same carboxy-terminal region and only differ in length due to their amino-terminal regions. As a result, the LHBs contain the preS1 + preS2 + S [389 or 400 amino acid (aa) residues], MHBs contain the preS2 + S (281 aa residues), and SHBs contain the S domain (226 aa residues) alone[3,20,22] (Figure 1). Additionally, a truncated and mutated preS2/S (the LHBs and truncated MHBs) can be produced by integrated viral sequences that are defective for replication[23,24]. LHBs, MHBs, and SHBs are important for HBV structure and life cycle. Besides mediating HBV entry through binding to HBV receptors, the sodium taurocholate co-transporting polypeptide (NTCP) on hepatocytes, via the preS1 2-48 aa domain (numbering for HBV-genotype D) and subsequent infection, LHBs are indispensable for the formation and budding of virions[3,25-29]. It has been proposed that LHBs rearrange their structure during the maturation of HBV virions and thereby regulate the release and infectivity of virions[30-32]. The exact role of MHBs in the HBV life cycle remains an enigma.

Figure 1 The transcription and expression of hepatitis B virus surface proteins. The three HBV surface proteins, LHBs, MHBs, and SHBs, are translated from two different mRNAs: LHBs are encoded by the preS1 promoter-initiated 2.4 kb subgenomic RNA; MHBs and SHBs are encoded by the preS2 promoter-initiated 2.1 kb subgenomic RNA. The 2.4 and 2.1 kb subgenomic RNAs share the same 3' end and only differ in length due to differences at the 5' end, which lead to different amino-terminal but identical carboxy-terminal regions of the three surface antigens. Therefore, LHBs contain preS1 + preS2 + S (389 or 400 aa residues), MHBs contain preS2 + S (281 aa residues), and SHBs contain the S domain (226 aa residues) alone. For mature/infectious virions, LHBs, MHBs, and SHBs are present in the envelopes at a ratio of approximately 1:1:4. In addition, the major fraction of SHBs forms subviral particles (filaments and spheres) together with the minor parts of LHBs and/or MHBs. HBV: Hepatitis B virus; LHBs: Large surface antigens; MHBs: Middle surface antigens; SHBs: Small surface antigens.

Early reports indicated that MHBs might be dispensable for HBV replication and virion formation; however, our data and those of other groups have shown that MHBs play a role in virion secretion[33-36]. Recently, MHBs were found to interact with ceruloplasmin and influence the production of extracellular virions[34]. As the predominant component of viral particles, including infectious virions and noninfectious subviral particles, SHBs are necessary for the production of virions and subviral particles[35]. For mature/infectious virions, LHBs, MHBs, and SHBs are present in the envelopes at a ratio of approximately 1:1:4[20]. Disturbance of this proportion impairs the production and release of virions[33]. For subviral particles, their amount outnumbers virions by 10000- to 1000000-fold, and the particles are detected serologically as HBsAg[11,37]. The secretion of subviral particles can also be suppressed by LHBs in a dose-dependent manner[38-41], thus promoting the S protein toward virion formation.

In addition, preS1, preS2, and S domains contain various B- and T-cell epitopes, which play an important role in inducing the host immune response[42,43]. The major hydrophilic region (MHR) between aa 100-169 of SHBs, especially the a-determinant located at aa 124-147, serves as the most important antigenic determinant in HBV surface proteins and is essential for HBsAg detection and HBV vaccine development[44,45]. Plasma-derived and recombinant HBsAg have been used for vaccine preparations and have induced strong specific and protective antibody responses in vaccines[46-48]. The presence of anti-HBs antibodies is considered to confer immunity against HBV infection. In contrast, a high quantity of circulating HBsAg in chronically HBV-infected patients is proposed as a factor leading to immune disturbance. Defective peripheral HBsAg-specific T cell responses in chronically infected patients were found to be correlated with serum HBsAg titers[49,50], suggesting that HBsAg overproduction influences the host's immune system in a way that is advantageous for the virus. *In vitro*, HBsAg can interfere with Toll-like receptor functions and trigger interleukin (IL)-10 production in Kuppfer cells[51-54]. Recently, published data has suggested that HBsAg may facilitate the induction of myeloid-derived suppressor cells in chronically HBV-infected patients[55]. HBsAg is also associated with the induction of regulatory T cells, as shown in HBV mouse models[56,57]. Thus, HBsAg is not only a structural component of virions and subviral

particles, but it also serves as an important immune modulator.

DEFECTIVE SURFACE ANTIGEN MUTATION AND HBV BIOLOGY

HBsAg mutants were first identified in individuals vaccinated against HBV but who were infected despite the presence of protective anti-HBs antibodies[58]. Those "immune escape" mutants with aa substitutions within a-determinants were found in different clinical settings, including vaccines, transplant patients receiving hyperimmunoglobulins, and immunocompromised patients with HBV reactivation[59-61]. Such mutant HBsAg commonly showed reduced binding to anti-HBs antibodies and decreased reactivity in established HBsAg detection assays[59-65]. The most widely known mutation is the sG145R mutation, which has been shown to be replication competent, may persist stably over time, and may be transmitted vertically or horizontally[66-69]. The sG145R mutation induces a strongly impaired anti-HBs antibody response, which could not efficiently clear HBsAg in an HBV hydrodynamic injection mouse model[70]. A similar result was also observed for another immune escape mutation, sK122I, indicating that such a defective surface antigen mutation may impair HBsAg neutralization and clearance during HBV infection. In addition, sG145R, sK122I, and other immune escape mutants occurring in the a-determinant of SHBs, such as the sT123N mutation, could affect HBsAg secretion[70-73].

Recently, chronically HBV-infected patients routinely received antiviral therapy based on nucleotide analogs[74]. Treatment with first-generation drugs, such as famciclovir and adeforvir, resulted in the emergence of drug-resistant HBV mutants, with aa substitutions within the HBV polymerase domain[75]. Some drug-resistant mutations occurring in the viral polymerase may lead to a stop codon mutation in the overlapping surface gene, cause intracellular retention of surface proteins, and result in secretion defects of viral particles, such as rtA181T/sW172*, rtM204I/sW196*, and rtV191I/sW182*, as shown in previous reports[76-78] and in our unpublished data. The primary sW182* mutation has also been identified in CHB patients. It was found to induce retention of the truncated S protein in the perinuclear endoplasmic reticulum (ER) and was associated with lower HBV transcript levels owing to decreased stability, but without impact on HBV replication[79].

Defective surface antigen mutations have been frequently detected in chronic HBV infection[16,71-73,80,81], in which deletions in the preS domains are the most common mutations[80,81]. Deletions in the preS domains are often clustered at the 3' end of preS1 and the 5' end of preS2[11,19,81-83]. Given that the preS2/S promoter is situated within the preS1 domain[11], deletions at the 3' end of the preS1 may reduce MHBs and SHBs expression at the transcriptional level. Deletions at the

5' end of the preS2 may remove the N-terminal preS2 domain, including the start codon of preS2 in the MHBs protein, leading to an impaired or complete loss of MHBs expression[84]. These changes may disrupt the proper LHBs, MHBs, and SHBs ratio in the envelopes of virions. In addition, the junction between the preS1 and preS2 domain is required for virion formation[32]. For these reasons, preS deletions may potentially affect virion assembly, stability, or infectivity.

A large amount of evidence has demonstrated that DHBV envelope proteins can regulate cccDNA formation and amplification[85,86]. Infection of envelope protein-deficient recombinant DHBV results in more cccDNA accumulation[85,87,88]. Similarly, deficiencies in HBV envelope proteins can modestly increase the cccDNA level and result in a dramatic accumulation of deproteinized rcDNA[89-91]. It has been demonstrated that preS/S mutants with surface antigen secretion deficiency isolated from patients can lead to an increased accumulation of cccDNA molecules in the nuclei[79]. Therefore, defective surface antigen mutation may affect cccDNA synthesis and amplification.

DEFECTIVE SURFACE ANTIGEN MUTATIONS AND THE HOST

Defective surface antigen mutations have been found in acute hepatitis B infection, chronic hepatitis B infection, and occult HBV infection and are associated with advanced liver disease, including liver cirrhosis, fulminant hepatitis B, and HCC[15,82,92-105]. It has been questioned whether HBV mutants arise due to viral adaptation to inflammation and decreased liver function or, alternatively, causally contribute to liver pathogenesis. The mechanism of defective surface antigen mutations to HCC development has been widely elucidated[11,23,41,106-111]. Here, we will emphasize in our discussion the relationship between defective surface antigen mutations and fulminant hepatitis B, as well as occult HBV infection.

Defective surface antigen expression and fulminant hepatitis

There is increasing evidence that defective surface antigen expression may play a role in the pathogenesis of fulminant hepatitis (FH). preS deletions, particularly those unable to synthesize the MHBs protein, have been associated with cases of FH[16,71,112,113], suggesting the potential pathogenic role of preS deletions. A mutation in the CAAT element of the S promoter has been found in the HBV genome isolated from an FH patient. This mutation led to excessive LHBs expression over MHBs and SHBs proteins and resulted in virus retention and misassembly[114-116]. Obviously, the accumulation of LHBs may be due to hepatocyte injury, as shown in transgenic mice with LHBs expression[41]. One of our previous studies also identified deletions within the preS

regions from HBV strains isolated from a patient with HBV-associated FH[84]. In addition, a hepatitis B immune globulin (HBIG)-escape mutant sG145R on the HBsAg, causing 30% inhibition of virion secretion, has been identified from a study on FH strains, suggesting the potential role of defective surface antigen expression in the fulminant clinical course of HBV infection[71].

Mechanistically, defective surface antigen expression, such as specific mutations in the preS/S gene, may lead to secretion defects of viral proteins and particles, resulting in an accumulation of viral products in the ER of hepatocytes and causing ER stress and hepatocyte injury[16]. Subsequently, autophagy may be triggered[117-125] and thus enhance HBV replication[126,127]. Consistent with this speculation, it has been demonstrated that defective surface antigen expression may increase the replication capability of HBV, albeit the mechanism is still undefined[71,84]. In addition, the deficiency of hepadnavirus envelope proteins can result in accumulation of cccDNA[85,87,88] or deproteinized rcDNA[89-91] and may ultimately cause death of the infected hepatocytes by a direct cytopathic effect[85,87,88]. Meanwhile, the increase of the cccDNA level may facilitate HBV replication. Both the defect in viral particle secretion and enhanced replication competence may contribute to the severity of fulminant hepatitis[128].

The adaptive immune response, particularly the cytotoxic T lymphocyte (CTL) response, plays a crucial role in viral clearance and disease pathogenesis of HBV infection[129-131]. Intracellular retention of HBV surface proteins was found to be associated with FH in a transgenic mouse model showing panlobular necrosis and hepatic failure by inducing the indirect cytotoxic activity of CTLs[132]. In this setting, intracellular accumulation of viral products due to defective surface antigen expression mutations may cause liver damage through abnormal activation of the CTL response. Consistently, we also observed significantly stronger intrahepatic CTL responses and antibody responses specific to secretion-deficient HBsAg due to preS deletions[84]. A preS deletion mutant was found in a patient with acute exacerbation of liver diseases, along with wild-type HBV genomes. The co-existence of deletion mutants and wild-type HBV apparently allows the complementation and enhancement of HBV genome replication in hepatocytes. In an HBV mouse model, co-replication of a deletion mutant and wild-type HBV induced higher cellular and humoral immunity. Our findings further suggested the proposed role of HBV variants in the immunopathogenesis of HBV infection. Moreover, the mutations associated with defective surface antigen expression, such as deletion or missense mutation of the preS2 ATG codon, can cause deletions or alterations of B- and T-cell epitopes located in preS1 and preS2 proteins. Considering that M protein-specific T- and B-cell immunities are important early events in the host immune response to HBV infection[43], these mutations may lead to an immune evasion

and thus likely favor a more severe clinical course of infection[14,133]. In chronic HBV infection, high HBV replication levels were found to be associated with lower cellular immune responses to HBV; however, massive infiltration of unspecific immune cells occurred within the liver, accompanied by severe liver damage[134-136]. Thus, the presence of these mutations, including aa substitutions at the immunodominant epitopes for B or T cell recognition, may contribute to the spread of highly replicative escape mutants. It may also facilitate the development of fulminant hepatitis in chronically HBV-infected patients and heavily immunocompromised patients, like those with human immunodeficiency virus (HIV) co-infection[137] (Figure 2).

Defective surface antigen expression and occult hepatitis B virus infection

Occult HBV infection (OBI) is characterized by the presence of very low levels of HBV DNA in the plasma and/or liver of individuals negative for HBV surface antigen (HBsAg) and positive/negative for antibodies to the hepatitis core antigen (anti-HBc)[45,138,139]. OBI harbors the potential risk of HBV transmission through blood transfusion, organ transplantation, and hemodialysis as well as from occult infection or HBsAg-positive mothers to newborns[45]. The persistence of OBI may lead to the development of cirrhosis and HCC[45,140-145]. The reactivation of OBI can occur in patients following chemotherapy, immunosuppressive therapy, and after transplantation as well as in patients co-infected with HIV or hepatitis C virus (HCV)[45,146,147], which can result in the development of fulminant hepatitis and death[139,148-153].

Defective surface antigen expression mutations may be associated with OBI. Point mutations and deletions as well as insertion mutations are commonly encountered in OBI, in which mutations in the *preS/S* gene are the most extensively studied[45]. High frequencies of MHR mutations, including those mutations within and outside of the a-determinant, have been observed in OBI strains of individuals[154-158]. *In vitro* and *in vivo* experiments have demonstrated that these MHR mutations can significantly decrease the detection sensitivity of commercial HBsAg immunoassays and impair virion and/or S protein secretion[156]. preS/S mutations with deletions covering the preS1 and preS2/S promoters, preS1 region, and preS2 region have been frequently reported in OBI. This can alter the transcription of 2.4 kb and 2.1 kb HBV RNAs, expression of three envelope proteins, and the ratio of LHBs/MHBs/SHBs proteins[45]. preS/S insertions, such as 2-8 aa insertions between codons 121 and 124 located upstream of the a-determinant, have also been observed in OBI patients[159].

On one hand, these mutations associated with defective surface antigen expression can directly decrease the levels of surface antigens. On the other hand, these mutations can cause the retention and
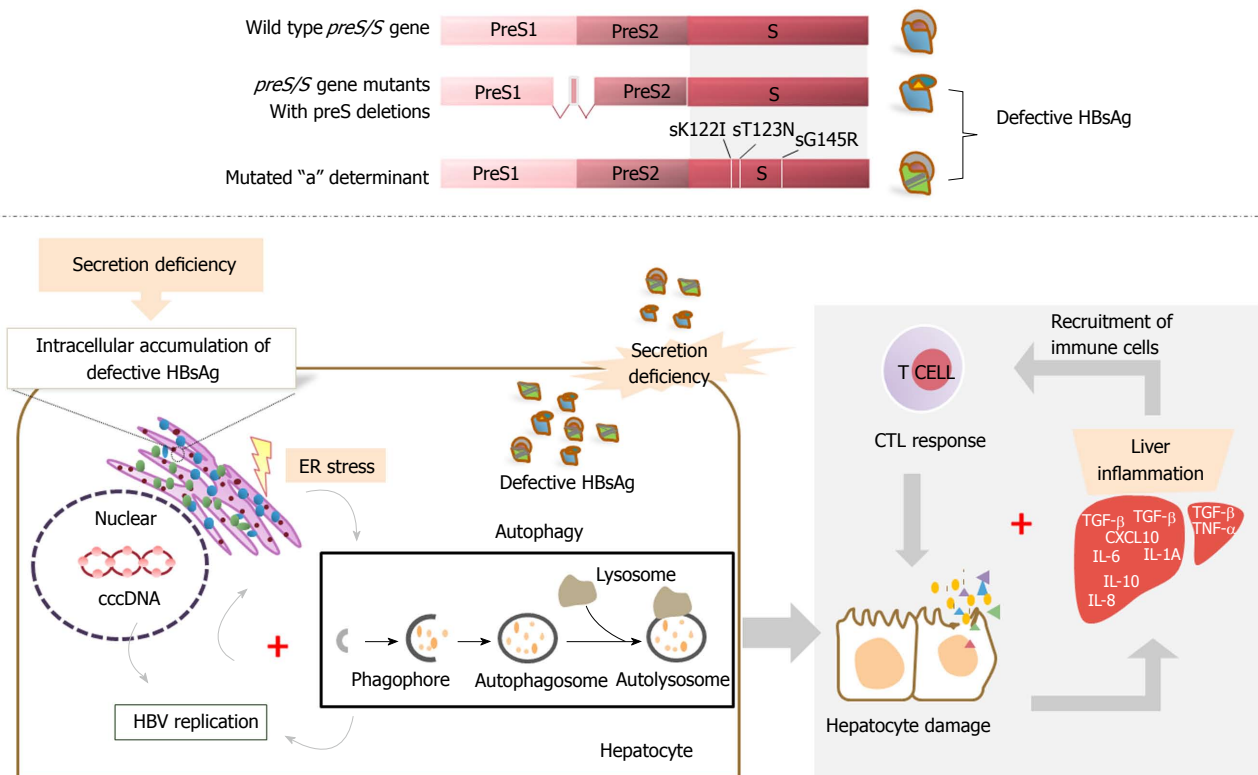
Figure 2 **The proposed pathogenic role of mutated secretion-defective hepatitis B surface antigen in fulminant hepatitis.** Defective surface antigens, such as preS deletions and mutations within the "a" determinant, may lead to secretion deficiency of HBsAg. Defective HBsAg can promote covalently closed circular DNA (cccDNA) synthesis and amplification, thus facilitating HBV replication. The intracellular accumulation of defective HBsAg can cause endoplasmic reticulum (ER) stress, subsequently trigger autophagy, and may further enhance HBV replication. The enhanced HBV replication, in turn, leads to accumulation of more defective HBsAg, possibly resulting in a positive feedback with unfavorable outcomes and hepatocyte damages. Inflammation may occur in the liver by recruiting immune cells. Cytotoxic T lymphocyte (CTL) response may be abnormally activated and damage infected hepatocytes, contributing to the progression of fulminant hepatitis. HBsAg: Hepatitis B surface antigen.

accumulation of HBsAg within cells and impair the secretion of HBsAg by altering the ratio of LHBs/MHBs/SHBs proteins[72,73,160,161]. Therefore, circulating HBsAg levels are low in the peripheral blood. Moreover, it is well documented that neutralizing antibodies produced during natural infection, or following active or passive immunization against HBV, are targeted to the conformational epitopes of the a-determinant[162]. Hence, single or multiple mutations occurring within this region can lead to conformational changes with impaired antigenicity[72,160]. A recent report has identified novel SHBs mutations outside the MHR from untreated CHB patients. These mutations impaired virion secretion and caused lower binding affinity to antibodies used for HBsAg immunoassays[163]. For these reasons, the mutations can render HBsAg undetectable or poorly detected by immunoassays based on monoclonal antibodies against wild-type virus[60,62,65,164], contributing to some cases of OBI[165-170] (Figure 3).

PERSPECTIVES

Defective surface antigen expression has been well documented to be relevant for the progression of HBV-associated liver diseases, such as HCC. However, the role of defective surface antigen expression in FH still needs to be clarified in future research, particularly, using *in vivo* models and in patients. The exact molecular mechanisms of how defective HBV surface antigens cause damage to hepatocytes and induce liver injury and subsequent pathogenic processes should be investigated. A deep understanding of the molecular mechanisms of HBV pathogenesis related to defective surface antigens is crucial to designing future therapeutic approaches. A critical question would be whether currently used nucleotide analogues (NAs) and interferon-based therapies can prevent such pathogenic processes. NAs are able to efficiently inhibit HBV DNA synthesis but not gene expression. Thus, HBV proteins, including surface antigens, are continuously produced under NA therapies. Another problem is the production of mutated HBV proteins from integrated HBV DNA, which are not controlled by NA therapies at all. Thus, specific interventions may be required to block the pathogenic potential of HBV proteins, besides efficient inhibition of HBV DNA synthesis. RNA silencing may be a suitable choice to achieve this goal[5,6,171,172].

An additional issue to be addressed is whether the defective surface antigen-related mutations may represent novel biomarkers of OBI. With improvement of HBV antigen and DNA detection assays, OBI will likely be easier to diagnose in the future. However,
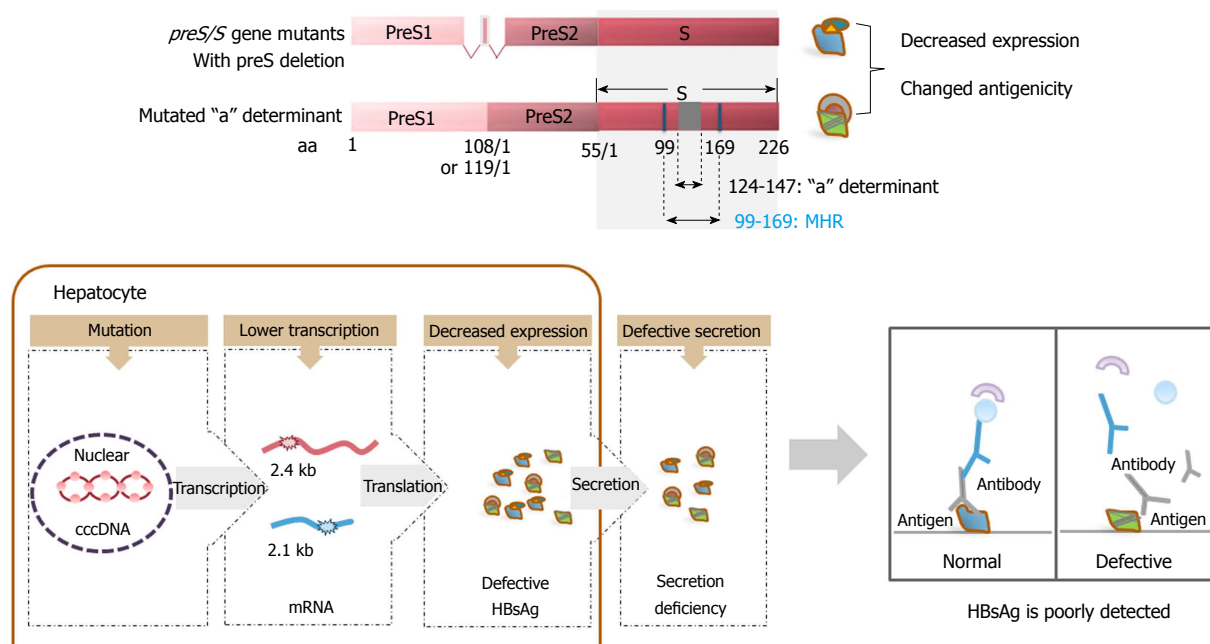
Figure 3 The relationship between the expression of defective surface antigens and occult hepatitis B virus infection. Surface antigen mutations, such as preS deletions, can impair the transcription of 2.4 and 2.1 kb HBV RNAs, leading to decreased levels of three HBV surface proteins. In addition, defective surface antigens with preS deletions and mutations within the "a" determinant are secretion deficient. Single or multiple mutations occurring within the MHR between the aa residues 99-169 of SHBs, especially those within the "a" determinant between aa 124-147, can lead to conformational changes of HBsAg. Mutated HBsAg is poorly detected by immunoassays based on monoclonal antibodies, contributing to some cases of OBI. OBI: Occult hepatitis B virus infection; HBsAg: Hepatitis B surface antigen.

the question remains whether OBI may be related to significant HBV pathogenesis and require therapeutic interventions, such as prophylaxis and antiviral therapy, to prevent HBV reactivation[173].

REFERENCES

1   **Ott JJ**, Stevens GA, Groeger J, Wiersma ST. Global epidemiology of hepatitis B virus infection: new estimates of age-specific HBsAg seroprevalence and endemicity. *Vaccine* 2012; **30**: 2212-2219 [PMID: 22273662 DOI: 10.1016/j.vaccine.2011.12.116]

2   **Polaris Observatory Collaborators.** Global prevalence, treatment, and prevention of hepatitis B virus infection in 2016: a modelling study. *Lancet Gastroenterol Hepatol* 2018; **3**: 383-403 [PMID: 29599078 DOI: 10.1016/S2468-1253(18)30056-6]

3   **Glebe D**, Bremer CM. The molecular virology of hepatitis B virus. *Semin Liver Dis* 2013; **33**: 103-112 [PMID: 23749666 DOI: 10.1055/s-0033-1345717]

4   **Bernal W**, Auzinger G, Dhawan A, Wendon J. Acute liver failure. *Lancet* 2010; **376**: 190-201 [PMID: 20638564 DOI: 10.1016/S0140-6736(10)60274-7]

5   **Lok AS**, Zoulim F, Dusheiko G, Ghany MG. Hepatitis B cure: From discovery to regulatory approval. *Hepatology* 2017; **66**: 1296-1313 [PMID: 28762522 DOI: 10.1002/hep.29323]

6   **Lok AS**, Zoulim F, Dusheiko G, Ghany MG. Hepatitis B cure: From discovery to regulatory approval. *J Hepatol* 2017; **67**: 847-861 [PMID: 28778687 DOI: 10.1016/j.jhep.2017.05.008]

7   **Kosinska AD**, Liu J, Lu M, Roggendorf M. Therapeutic vaccination and immunomodulation in the treatment of chronic hepatitis B: preclinical studies in the woodchuck. *Med Microbiol Immunol* 2015; **204**: 103-114 [PMID: 25535101 DOI: 10.1007/s00430-014-0379-5]

8   **Seeger C**, Mason WS. Molecular biology of hepatitis B virus infection. *Virology* 2015; **479-480**: 672-686 [PMID: 25759099 DOI: 10.1016/j.virol.2015.02.031]

9   **Nassal M**. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. *Gut* 2015; **64**: 1972-1984 [PMID: 26048673 DOI: 10.1136/gutjnl-2015-309809]

10 **Seeger C**. Control of viral transcripts as a concept for future HBV therapies. *Curr Opin Virol* 2018; **30**: 18-23 [PMID: 29453098 DOI: 10.1016/j.coviro.2018.01.009]

11 **Tong S**, Revill P. Overview of hepatitis B viral replication and genetic variability. *J Hepatol* 2016; **64**: S4-S16 [PMID: 27084035 DOI: 10.1016/j.jhep.2016.01.027]

12 **Gerlich WH**. Medical virology of hepatitis B: how it began and where we are now. *Virol J* 2013; **10**: 239 [PMID: 23870415 DOI: 10.1186/1743-422X-10-239]

13 **Lin CL**, Kao JH. Hepatitis B virus genotypes and variants. *Cold Spring Harb Perspect Med* 2015; **5**: a021436 [PMID: 25934462 DOI: 10.1101/cshperspect.a021436]

14 **Kay A**, Zoulim F. Hepatitis B virus genetic variability and evolution. *Virus Res* 2007; **127**: 164-176 [PMID: 17383765 DOI: 10.1016/j.virusres.2007.02.021]

15 **Kao JH**, Chen PJ, Chen DS. Recent advances in the research of hepatitis B virus-related hepatocellular carcinoma: epidemiologic and molecular biological aspects. *Adv Cancer Res* 2010; **108**: 21-72 [PMID: 21034965 DOI: 10.1016/B978-0-12-380888-2.00002-9]

16 **Pollicino T**, Cacciola I, Saffioti F, Raimondo G. Hepatitis B virus PreS/S gene variants: pathobiology and clinical implications. *J Hepatol* 2014; **61**: 408-417 [PMID: 24801416 DOI: 10.1016/j.jhep.2014.04.041]

17 **Shen T**, Yan XM. Hepatitis B virus genetic mutations and evolution in liver diseases. *World J Gastroenterol* 2014; **20**: 5435-5441 [PMID: 24833874 DOI: 10.3748/wjg.v20.i18.5435]

18 **Zhang ZH**, Wu CC, Chen XW, Li X, Li J, Lu MJ. Genetic variation of hepatitis B virus and its significance for pathogenesis. *World J Gastroenterol* 2016; **22**: 126-144 [PMID: 26755865 DOI: 10.3748/wjg.v22.i1.126]

19 **Chen BF**, Liu CJ, Jow GM, Chen PJ, Kao JH, Chen DS. High prevalence and mapping of pre-S deletion in hepatitis B virus carriers with progressive liver diseases. *Gastroenterology* 2006; **130**: 1153-1168 [PMID: 16618410 DOI: 10.1053/j.gastro.2006.01.011]

20   **Heermann KH**, Goldmann U, Schwartz W, Seyffarth T, Baumgarten H, Gerlich WH. Large surface proteins of hepatitis B virus containing the pre-s sequence. *J Virol* 1984; **52**: 396-402 [PMID: 6492255]

21   **Cattaneo R**, Will H, Hernandez N, Schaller H. Signals regulating hepatitis B surface antigen transcription. *Nature* 1983; **305**: 336-338 [PMID: 6621688]

22   **Stibbe W**, Gerlich WH. Structural relationships between minor and major proteins of hepatitis B surface antigen. *J Virol* 1983; **46**: 626-628 [PMID: 6842680]

23   **Schlüter V**, Meyer M, Hofschneider PH, Koshy R, Caselmann WH. Integrated hepatitis B virus X and 3' truncated preS/S sequences derived from human hepatomas encode functionally active transactivators. *Oncogene* 1994; **9**: 3335-3344 [PMID: 7936659]

24   **Li YW**, Yang FC, Lu HQ, Zhang JS. Hepatocellular carcinoma and hepatitis B surface protein. *World J Gastroenterol* 2016; **22**: 1943-1952 [PMID: 26877602 DOI: 10.3748/wjg.v22.i6.1943]

25   **Le Seyec J**, Chouteau P, Cannie I, Guguen-Guillouzo C, Gripon P. Infection process of the hepatitis B virus depends on the presence of a defined sequence in the pre-S1 domain. *J Virol* 1999; **73**: 2052-2057 [PMID: 9971786]

26   **Schulze A**, Gripon P, Urban S. Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. *Hepatology* 2007; **46**: 1759-1768 [PMID: 18046710 DOI: 10.1002/hep.21896]

27   **Glebe D**, Urban S, Knoop EV, Cag N, Krass P, Grün S, Bulavaite A, Sasnauskas K, Gerlich WH. Mapping of the hepatitis B virus attachment site by use of infection-inhibiting preS1 lipopeptides and tupaia hepatocytes. *Gastroenterology* 2005; **129**: 234-245 [PMID: 16012950]

28   **Glebe D**, Aliakbari M, Krass P, Knoop EV, Valerius KP, Gerlich WH. Pre-s1 antigen-dependent infection of Tupaia hepatocyte cultures with human hepatitis B virus. *J Virol* 2003; **77**: 9511-9521 [PMID: 12915565]

29   **Yan H**, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, Fu L, Song M, Chen P, Gao W, Ren B, Sun Y, Cai T, Feng X, Sui J, Li W. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *Elife* 2012; **1**: e00049 [PMID: 23150796 DOI: 10.7554/eLife.00049]

30   **Ostapchuk P**, Hearing P, Ganem D. A dramatic shift in the transmembrane topology of a viral envelope glycoprotein accompanies hepatitis B viral morphogenesis. *EMBO J* 1994; **13**: 1048-1057 [PMID: 8131739]

31   **Bruss V**, Lu X, Thomssen R, Gerlich WH. Post-translational alterations in transmembrane topology of the hepatitis B virus large envelope protein. *EMBO J* 1994; **13**: 2273-2279 [PMID: 8194518]

32   **Bruss V**. Hepatitis B virus morphogenesis. *World J Gastroenterol* 2007; **13**: 65-73 [PMID: 17206755]

33   **Garcia T**, Li J, Sureau C, Ito K, Qin Y, Wands J, Tong S. Drastic reduction in the production of subviral particles does not impair hepatitis B virus virion secretion. *J Virol* 2009; **83**: 11152-11165 [PMID: 19706705 DOI: 10.1128/JVI.00905-09]

34   **Zhao K**, Wu C, Yao Y, Cao L, Zhang Z, Yuan Y, Wang Y, Pei R, Chen J, Hu X, Zhou Y, Lu M, Chen X. Ceruloplasmin inhibits the production of extracellular hepatitis B virions by targeting its middle surface protein. *J Gen Virol* 2017; **98**: 1410-1421 [PMID: 28678687 DOI: 10.1099/jgv.0.000794]

35   **Bruss V**, Ganem D. The role of envelope proteins in hepatitis B virus assembly. *Proc Natl Acad Sci USA* 1991; **88**: 1059-1063 [PMID: 1992457]

36   **Fernholz D**, Galle PR, Stemler M, Brunetto M, Bonino F, Will H. Infectious hepatitis B virus variant defective in pre-S2 protein expression in a chronic carrier. *Virology* 1993; **194**: 137-148 [PMID: 8480417 DOI: 10.1006/viro.1993.1243]

37   **Ganem D**, Prince AM. Hepatitis B virus infection--natural history and clinical consequences. *N Engl J Med* 2004; **350**: 1118-1129 [PMID: 15014185 DOI: 10.1056/NEJMra031087]

38   **Persing DH**, Varmus HE, Ganem D. Inhibition of secretion of hepatitis B surface antigen by a related presurface polypeptide.

Science 1986; **234**: 1388-1391 [PMID: 3787251]

39   **Ou JH**, Rutter WJ. Regulation of secretion of the hepatitis B virus major surface antigen by the preS-1 protein. *J Virol* 1987; **61**: 782-786 [PMID: 3806798]

40   **Chisari FV**, Filippi P, McLachlan A, Milich DR, Riggs M, Lee S, Palmiter RD, Pinkert CA, Brinster RL. Expression of hepatitis B virus large envelope polypeptide inhibits hepatitis B surface antigen secretion in transgenic mice. *J Virol* 1986; **60**: 880-887 [PMID: 3783819]

41   **Chisari FV**, Filippi P, Buras J, McLachlan A, Popper H, Pinkert CA, Palmiter RD, Brinster RL. Structural and pathological effects of synthesis of hepatitis B virus large envelope polypeptide in transgenic mice. *Proc Natl Acad Sci USA* 1987; **84**: 6909-6913 [PMID: 3477814]

42   **Nayersina R**, Fowler P, Guilhot S, Missale G, Cerny A, Schlicht HJ, Vitiello A, Chesnut R, Person JL, Redeker AG, Chisari FV. HLA A2 restricted cytotoxic T lymphocyte responses to multiple hepatitis B surface antigen epitopes during hepatitis B virus infection. *J Immunol* 1993; **150**: 4659-4671 [PMID: 7683326]

43   **Chisari FV**, Ferrari C. Hepatitis B virus immunopathogenesis. *Annu Rev Immunol* 1995; **13**: 29-60 [PMID: 7612225 DOI: 10.1146/annurev.iy.13.040195.000333]

44   **Coppola N**, Onorato L, Minichini C, Di Caprio G, Starace M, Sagnelli C, Sagnelli E. Clinical significance of hepatitis B surface antigen mutants. *World J Hepatol* 2015; **7**: 2729-2739 [PMID: 26644816 DOI: 10.4254/wjh.v7.i27.2729]

45   **Zhu HL**, Li X, Li J, Zhang ZH. Genetic variation of occult hepatitis B virus infection. *World J Gastroenterol* 2016; **22**: 3531-3546 [PMID: 27053845 DOI: 10.3748/wjg.v22.i13.3531]

46   **Maupas P**, Chiron JP, Barin F, Coursaget P, Goudeau A, Perrin J, Denis F, Mar ID. Efficacy of hepatitis B vaccine in prevention of early HBsAg carrier state in children. Controlled trial in an endemic area (Senegal). *Lancet* 1981; **1**: 289-292 [PMID: 6109938]

47   **Szmuness W**, Stevens CE, Harley EJ, Zang EA, Oleszko WR, William DC, Sadovsky R, Morrison JM, Kellner A. Hepatitis B vaccine: demonstration of efficacy in a controlled clinical trial in a high-risk population in the United States. *N Engl J Med* 1980; **303**: 833-841 [PMID: 6997738 DOI: 10.1056/NEJM198010093031501]

48   **Valenzuela P**, Medina A, Rutter WJ, Ammerer G, Hall BD. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature* 1982; **298**: 347-350 [PMID: 7045698]

49   **Reignat S**, Webster GJ, Brown D, Ogg GS, King A, Seneviratne SL, Dusheiko G, Williams R, Maini MK, Bertoletti A. Escaping high viral load exhaustion: CD8 cells with altered tetramer binding in chronic hepatitis B virus infection. *J Exp Med* 2002; **195**: 1089-1101 [PMID: 11994415]

50   **Guidotti LG**, Isogawa M, Chisari FV. Host-virus interactions in hepatitis B virus infection. *Curr Opin Immunol* 2015; **36**: 61-66 [PMID: 26186123 DOI: 10.1016/j.coi.2015.06.016]

51   **Wang S**, Chen Z, Hu C, Qian F, Cheng Y, Wu M, Shi B, Chen J, Hu Y, Yuan Z. Hepatitis B virus surface antigen selectively inhibits TLR2 ligand-induced IL-12 production in monocytes/macrophages by interfering with JNK activation. *J Immunol* 2013; **190**: 5142-5151 [PMID: 23585678 DOI: 10.4049/jimmunol.1201625]

52   **Liu J**, Yu Q, Wu W, Huang X, Broering R, Werner M, Roggendorf M, Yang D, Lu M. TLR2 Stimulation Strengthens Intrahepatic Myeloid-Derived Cell-Mediated T Cell Tolerance through Inducing Kupffer Cell Expansion and IL-10 Production. *J Immunol* 2018; **200**: 2341-2351 [PMID: 29459406 DOI: 10.4049/jimmunol.1700540]

53   **Jiang M**, Broering R, Trippler M, Poggenpohl L, Fiedler M, Gerken G, Lu M, Schlaak JF. Toll-like receptor-mediated immune responses are attenuated in the presence of high levels of hepatitis B virus surface antigen. *J Viral Hepat* 2014; **21**: 860-872 [PMID: 24498958 DOI: 10.1111/jvh.12216]

54   **Wu J**, Meng Z, Jiang M, Pei R, Trippler M, Broering R, Bucchi A, Sowa JP, Dittmer U, Yang D, Roggendorf M, Gerken G, Lu M, Schlaak JF. Hepatitis B virus suppresses toll-like receptor-mediated innate immune responses in murine parenchymal and nonparenchymal liver cells. *Hepatology* 2009; **49**: 1132-1140

[PMID: 19140219 DOI: 10.1002/hep.22751]

55   **Fang Z**, Li J, Yu X, Zhang D, Ren G, Shi B, Wang C, Kosinska AD, Wang S, Zhou X, Kozlowski M, Hu Y, Yuan Z. Polarization of Monocytic Myeloid-Derived Suppressor Cells by Hepatitis B Surface Antigen Is Mediated via ERK/IL-6/STAT3 Signaling Feedback and Restrains the Activation of T Cells in Chronic Hepatitis B Virus Infection. *J Immunol* 2015; **195**: 4873-4883 [PMID: 26416274 DOI: 10.4049/jimmunol.1501362]

56   **Dietze KK**, Schimmer S, Kretzmer F, Wang J, Lin Y, Huang X, Wu W, Wang B, Lu M, Dittmer U, Yang D, Liu J. Characterization of the Treg Response in the Hepatitis B Virus Hydrodynamic Injection Mouse Model. *PLoS One* 2016; **11**: e0151717 [PMID: 26986976 DOI: 10.1371/journal.pone.0151717]

57   **Kosinska AD**, Pishraft-Sabet L, Wu W, Fang Z, Lenart M, Chen J, Dietze KK, Wang C, Kemper T, Lin Y, Yeh SH, Liu J, Dittmer U, Yuan Z, Roggendorf M, Lu M. Low hepatitis B virus-specific T-cell response in males correlates with high regulatory T-cell numbers in murine models. *Hepatology* 2017; **66**: 69-83 [PMID: 28295453 DOI: 10.1002/hep.29155]

58   **Zanetti AR**, Tanzi E, Manzillo G, Maio G, Sbreglia C, Caporaso N, Thomas H, Zuckerman AJ. Hepatitis B variant in Europe. *Lancet* 1988; **2**: 1132-1133 [PMID: 2460710]

59   **Carman WF**, Zanetti AR, Karayiannis P, Waters J, Manzillo G, Tanzi E, Zuckerman AJ, Thomas HC. Vaccine-induced escape mutant of hepatitis B virus. *Lancet* 1990; **336**: 325-329 [PMID: 1697396]

60   **Cooreman MP**, van Roosmalen MH, te Morsche R, Sünnen CM, de Ven EM, Jansen JB, Tytgat GN, de Wit PL, Paulij WP. Characterization of the reactivity pattern of murine monoclonal antibodies against wild-type hepatitis B surface antigen to G145R and other naturally occurring "a" loop escape mutations. *Hepatology* 1999; **30**: 1287-1292 [PMID: 10534351 DOI: 10.1002/hep.510300508]

61   **Cooreman MP**, Leroux-Roels G, Paulij WP. Vaccine- and hepatitis B immune globulin-induced escape mutations of hepatitis B virus surface antigen. *J Biomed Sci* 2001; **8**: 237-247 [PMID: 11385295 DOI: 10.1159/000054039]

62   **Chiou HL**, Lee TS, Kuo J, Mau YC, Ho MS. Altered antigenicity of 'a' determinant variants of hepatitis B virus. *J Gen Virol* 1997; **78** (Pt 10): 2639-2645 [PMID: 9349486 DOI: 10.1099/0022-1317-78-10-2639]

63   **Günther S**, Fischer L, Pult I, Sterneck M, Will H. Naturally occurring variants of hepatitis B virus. *Adv Virus Res* 1999; **52**: 25-137 [PMID: 10384235]

64   **Lu M**, Lorentz T. De novo infection in a renal transplant recipient caused by novel mutants of hepatitis B virus despite the presence of protective anti-hepatitis B surface antibody. *J Infect Dis* 2003; **187**: 1323-1326 [PMID: 12696014 DOI: 10.1086/373902]

65   **Seddigh-Tonekaboni S**, Waters JA, Jeffers S, Gehrke R, Ofenloch B, Horsch A, Hess G, Thomas HC, Karayiannis P. Effect of variation in the common "a" determinant on the antigenicity of hepatitis B surface antigen. *J Med Virol* 2000; **60**: 113-121 [PMID: 10596008]

66   **Ghany MG**, Ayola B, Villamil FG, Gish RG, Rojter S, Vierling JM, Lok AS. Hepatitis B virus S mutants in liver transplant recipients who were reinfected despite hepatitis B immune globulin prophylaxis. *Hepatology* 1998; **27**: 213-222 [PMID: 9425940 DOI: 10.1002/hep.510270133]

67   **Hsu HY**, Chang MH, Ni YH, Lin HH, Wang SM, Chen DS. Surface gene mutants of hepatitis B virus in infants who develop acute or chronic infections despite immunoprophylaxis. *Hepatology* 1997; **26**: 786-791 [PMID: 9303514 DOI: 10.1002/hep.510260336]

68   **Okamoto H**, Yano K, Nozaki Y, Matsui A, Miyazaki H, Yamamoto K, Tsuda F, Machida A, Mishiro S. Mutations within the S gene of hepatitis B virus transmitted from mothers to babies immunized with hepatitis B immune globulin and vaccine. *Pediatr Res* 1992; **32**: 264-268 [PMID: 1383917 DOI: 10.1203/00006450-199209000-00002]

69   **Protzer-Knolle U**, Naumann U, Bartenschlager R, Berg T, Hopf U, Meyer zum Büschenfelde KH, Neuhaus P, Gerken G. Hepatitis B virus with antigenically altered hepatitis B surface antigen is selected by high-dose hepatitis B immune globulin after liver transplantation. *Hepatology* 1998; **27**: 254-263 [PMID: 9425945 DOI: 10.1002/hep.510270138]

70   **Wu C**, Deng W, Deng L, Cao L, Qin B, Li S, Wang Y, Pei R, Yang D, Lu M, Chen X. Amino acid substitutions at positions 122 and 145 of hepatitis B virus surface antigen (HBsAg) determine the antigenicity and immunogenicity of HBsAg and influence in vivo HBsAg clearance. *J Virol* 2012; **86**: 4658-4669 [PMID: 22301154 DOI: 10.1128/JVI.06353-11]

71   **Kalinina T**, Riu A, Fischer L, Will H, Sterneck M. A dominant hepatitis B virus population defective in virus secretion because of several S-gene mutations from a patient with fulminant hepatitis. *Hepatology* 2001; **34**: 385-394 [PMID: 11481624 DOI: 10.1053/jhep.2001.26516]

72   **Wu C**, Zhang X, Tian Y, Song J, Yang D, Roggendorf M, Lu M, Chen X. Biological significance of amino acid substitutions in hepatitis B surface antigen (HBsAg) for glycosylation, secretion, antigenicity and immunogenicity of HBsAg and hepatitis B virus replication. *J Gen Virol* 2010; **91**: 483-492 [PMID: 19812261 DOI: 10.1099/vir.0.012740-0]

73   **Li S**, Zhao K, Liu S, Wu C, Yao Y, Cao L, Hu X, Zhou Y, Wang Y, Pei R, Lu M, Chen X. HBsAg sT123N mutation induces stronger antibody responses to HBsAg and HBcAg and accelerates in vivo HBsAg clearance. *Virus Res* 2015; **210**: 119-125 [PMID: 26260331 DOI: 10.1016/j.virusres.2015.08.004]

74   **Zoulim F**, Durantel D. Antiviral therapies and prospects for a cure of chronic hepatitis B. *Cold Spring Harb Perspect Med* 2015; **5** [PMID: 25833942 DOI: 10.1101/cshperspect.a021501]

75   **Zoulim F**, Locarnini S. Hepatitis B virus resistance to nucleos(t)ide analogues. *Gastroenterology* 2009; **137**: 1593-1608.e1-2 [PMID: 19737565 DOI: 10.1053/j.gastro.2009.08.063]

76   **Warner N**, Locarnini S. The antiviral drug selected hepatitis B virus rtA181T/sW172* mutant has a dominant negative secretion defect and alters the typical profile of viral rebound. *Hepatology* 2008; **48**: 88-98 [PMID: 18537180 DOI: 10.1002/hep.22295]

77   **Ahn SH**, Park YK, Park ES, Kim JH, Kim DH, Lim KH, Jang MS, Choe WH, Ko SY, Sung IK, Kwon SY, Kim KH. The impact of the hepatitis B virus polymerase rtA181T mutation on replication and drug resistance is potentially affected by overlapping changes in surface gene. *J Virol* 2014; **88**: 6805-6818 [PMID: 24696492 DOI: 10.1128/JVI.00635-14]

78   **Yeh CT**. Development of HBV S gene mutants in chronic hepatitis B patients receiving nucleotide/nucleoside analogue therapy. *Antivir Ther* 2010; **15**: 471-475 [PMID: 20516567 DOI: 10.3851/IMP1552]

79   **Pollicino T**, Amaddeo G, Restuccia A, Raffa G, Alibrandi A, Cutroneo G, Favaloro A, Maimone S, Squadrito G, Raimondo G. Impact of hepatitis B virus (HBV) preS/S genomic variability on HBV surface antigen and HBV DNA serum levels. *Hepatology* 2012; **56**: 434-443 [PMID: 22271491 DOI: 10.1002/hep.25592]

80   **Fernholz D**, Stemler M, Brunetto M, Bonino F, Will H. Replicating and virion secreting hepatitis B mutant virus unable to produce preS2 protein. *J Hepatol* 1991; **13** Suppl 4: S102-S104 [PMID: 1822500]

81   **Gerken G**, Kremsdorf D, Capel F, Petit MA, Dauguet C, Manns MP, Meyer zum Büschenfelde KH, Brechot C. Hepatitis B defective virus with rearrangements in the preS gene during chronic HBV infection. *Virology* 1991; **183**: 555-565 [PMID: 1853561]

82   **Sugauchi F**, Ohno T, Orito E, Sakugawa H, Ichida T, Komatsu M, Kuramitsu T, Ueda R, Miyakawa Y, Mizokami M. Influence of hepatitis B virus genotypes on the development of preS deletions and advanced liver disease. *J Med Virol* 2003; **70**: 537-544 [PMID: 12794715 DOI: 10.1002/jmv.10428]

83   **Chen CH**, Changchien CS, Lee CM, Hung CH, Hu TH, Wang JH, Wang JC, Lu SN. Combined mutations in pre-s/surface and core promoter/precore regions of hepatitis B virus increase the risk of hepatocellular carcinoma: a case-control study. *J Infect Dis* 2008; **198**: 1634-1642 [PMID: 18939932 DOI: 10.1086/592990]

84   **Cao L**, Wu C, Shi H, Gong Z, Zhang E, Wang H, Zhao K, Liu S, Li S, Gao X, Wang Y, Pei R, Lu M, Chen X. Coexistence of hepatitis

B virus quasispecies enhances viral replication and the ability to induce host antibody and cellular immune responses. *J Virol* 2014; **88**: 8656-8666 [PMID: 24850745 DOI: 10.1128/JVI.01123-14]

85 **Summers J**, Smith PM, Horwich AL. Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. *J Virol* 1990; **64**: 2819-2824 [PMID: 2335817]

86 **Lenhoff RJ**, Summers J. Coordinate regulation of replication and virus assembly by the large envelope protein of an avian hepadnavirus. *J Virol* 1994; **68**: 4565-4571 [PMID: 8207830]

87 **Lenhoff RJ**, Luscombe CA, Summers J. Acute liver injury following infection with a cytopathic strain of duck hepatitis B virus. *Hepatology* 1999; **29**: 563-571 [PMID: 9918936 DOI: 10.1002/hep.510290236]

88 **Summers J**, Smith PM, Huang MJ, Yu MS. Morphogenetic and regulatory effects of mutations in the envelope proteins of an avian hepadnavirus. *J Virol* 1991; **65**: 1310-1317 [PMID: 1995945]

89 **Gao W**, Hu J. Formation of hepatitis B virus covalently closed circular DNA: removal of genome-linked protein. *J Virol* 2007; **81**: 6164-6174 [PMID: 17409153 DOI: 10.1128/JVI.02721-06]

90 **Guo H**, Jiang D, Zhou T, Cuconati A, Block TM, Guo JT. Characterization of the intracellular deproteinized relaxed circular DNA of hepatitis B virus: an intermediate of covalently closed circular DNA formation. *J Virol* 2007; **81**: 12472-12484 [PMID: 17804499 DOI: 10.1128/JVI.01123-07]

91 **Lentz TB**, Loeb DD. Roles of the envelope proteins in the amplification of covalently closed circular DNA and completion of synthesis of the plus-strand DNA in hepatitis B virus. *J Virol* 2011; **85**: 11916-11927 [PMID: 21900164 DOI: 10.1128/JVI.05373-11]

92 **Mu SC**, Lin YM, Jow GM, Chen BF. Occult hepatitis B virus infection in hepatitis B vaccinated children in Taiwan. *J Hepatol* 2009; **50**: 264-272 [PMID: 19070923 DOI: 10.1016/j.jhep.2008.09.017]

93 **Chirara MM**, Chetsanga CJ. Variant of hepatitis B virus isolated in Zimbabwe. *J Med Virol* 1994; **42**: 73-78 [PMID: 8308523]

94 **Bowyer SM**, van Staden L, Kew MC, Sim JG. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. *J Gen Virol* 1997; **78** (Pt 7): 1719-1729 [PMID: 9225049 DOI: 10.1099/0022-1317-78-7-1719]

95 **Owiredu WK**, Kramvis A, Kew MC. Molecular analysis of hepatitis B virus genomes isolated from black African patients with fulminant hepatitis B. *J Med Virol* 2001; **65**: 485-492 [PMID: 11596083]

96 **Garfein RS**, Bower WA, Loney CM, Hutin YJ, Xia GL, Jawanda J, Groom AV, Nainan OV, Murphy JS, Bell BP. Factors associated with fulminant liver failure during an outbreak among injection drug users with acute hepatitis B. *Hepatology* 2004; **40**: 865-873 [PMID: 15382123 DOI: 10.1002/hep.20383]

97 **Liaw YF**, Leung N, Kao JH, Piratvisuth T, Gane E, Han KH, Guan R, Lau GK, Locarnini S; Chronic Hepatitis B Guideline Working Party of the Asian-Pacific Association for the Study of the Liver. Asian-Pacific consensus statement on the management of chronic hepatitis B: a 2008 update. *Hepatol Int* 2008; **2**: 263-283 [PMID: 19669255 DOI: 10.1007/s12072-008-9080-3]

98 **Shen T**, Yan XM, Zou YL, Gao JM, Dong H. Virologic characteristics of hepatitis B virus in patients infected via maternal-fetal transmission. *World J Gastroenterol* 2008; **14**: 5674-5682 [PMID: 18837083]

99 **Shen FC**, Su IJ, Wu HC, Hsieh YH, Yao WJ, Young KC, Chang TC, Hsieh HC, Tsai HN, Huang W. A pre-S gene chip to detect pre-S deletions in hepatitis B virus large surface antigen as a predictive marker for hepatoma risk in chronic hepatitis B virus carriers. *J Biomed Sci* 2009; **16**: 84 [PMID: 19751529 DOI: 10.1186/1423-0127-16-84]

100 **Shen T**, Yan XM, Zhang JP, Wang JL, Zuo RX, Li L, Wang LP. Evolution of Hepatitis B Virus in a Chronic HBV-Infected Patient over 2 Years. *Hepat Res Treat* 2011; **2011**: 939148 [PMID: 21785721 DOI: 10.1155/2011/939148]

101 **Heo NY**, Lee HC, Park YK, Park JW, Lim YS, Kim KM, Shim JH, Lee YJ. Lack of association between hepatitis B virus pre-S mutations and recurrence after surgical resection in hepatocellular carcinoma. *J Med Virol* 2013; **85**: 589-596 [PMID: 23296476 DOI: 10.1002/jmv.23502]

102 **Hung CH**, Chen CH, Lee CM, Hu TH, Lu SN, Wang JH, Huang CM. Role of viral genotypes and hepatitis B viral mutants in the risk of hepatocellular carcinoma associated with hepatitis B and C dual infection. *Intervirology* 2013; **56**: 316-324 [PMID: 23838434 DOI: 10.1159/000350738]

103 **Fan YF**, Lu CC, Chen WC, Yao WJ, Wang HC, Chang TT, Lei HY, Shiau AL, Su IJ. Prevalence and significance of hepatitis B virus (HBV) pre-S mutants in serum and liver at different replicative stages of chronic HBV infection. *Hepatology* 2001; **33**: 277-286 [PMID: 11124846 DOI: 10.1053/jhep.2001.21163]

104 **Chen CH**, Hung CH, Lee CM, Hu TH, Wang JH, Wang JC, Lu SN, Changchien CS. Pre-S deletion and complex mutations of hepatitis B virus related to advanced liver disease in HBeAg-negative patients. *Gastroenterology* 2007; **133**: 1466-1474 [PMID: 17915220 DOI: 10.1053/j.gastro.2007.09.002]

105 **Ghosh S**, Mondal RK, Banerjee P, Nandi M, Sarkar S, Das K, Santra A, Banerjee S, Chowdhury A, Datta S. Tracking the naturally occurring mutations across the full-length genome of hepatitis B virus of genotype D in different phases of chronic e-antigen-negative infection. *Clin Microbiol Infect* 2012; **18**: E412-E418 [PMID: 22827722 DOI: 10.1111/j.1469-0691.2012.03975.x]

106 **Xu Z**, Yen TS. Intracellular retention of surface protein by a hepatitis B virus mutant that releases virion particles. *J Virol* 1996; **70**: 133-140 [PMID: 8523517]

107 **Su IJ**, Wang LH, Hsieh WC, Wu HC, Teng CF, Tsai HW, Huang W. The emerging role of hepatitis B virus pre-S2 deletion mutant proteins in HBV tumorigenesis. *J Biomed Sci* 2014; **21**: 98 [PMID: 25316153 DOI: 10.1186/s12929-014-0098-7]

108 **Arbuthnot P**, Kew M. Hepatitis B virus and hepatocellular carcinoma. *Int J Exp Pathol* 2001; **82**: 77-100 [PMID: 11454100]

109 **Hildt E**, Hofschneider PH. The PreS2 activators of the hepatitis B virus: activators of tumour promoter pathways. *Recent Results Cancer Res* 1998; **154**: 315-329 [PMID: 10027012]

110 **Caselmann WH**, Meyer M, Kekulé AS, Lauer U, Hofschneider PH, Koshy R. A trans-activator function is generated by integration of hepatitis B virus preS/S sequences in human hepatocellular carcinoma DNA. *Proc Natl Acad Sci USA* 1990; **87**: 2970-2974 [PMID: 2158099]

111 **Lauer U**, Weiss L, Lipp M, Hofschneider PH, Kekulé AS. The hepatitis B virus preS2/St transactivator utilizes AP-1 and other transcription factors for transactivation. *Hepatology* 1994; **19**: 23-31 [PMID: 8276360]

112 **Sterneck M**, Günther S, Gerlach J, Naoumov NV, Santantonio T, Fischer L, Rogiers X, Greten H, Williams R, Will H. Hepatitis B virus sequence changes evolving in liver transplant recipients with fulminant hepatitis. *J Hepatol* 1997; **26**: 754-764 [PMID: 9126786]

113 **Pollicino T**, Zanetti AR, Cacciola I, Petit MA, Smedile A, Campo S, Sagliocca L, Pasquali M, Tanzi E, Longo G, Raimondo G. Pre-S2 defective hepatitis B virus infection in patients with fulminant hepatitis. *Hepatology* 1997; **26**: 495-499 [PMID: 9252165 DOI: 10.1002/hep.510260235]

114 **Bock CT**, Tillmann HL, Maschek HJ, Manns MP, Trautwein C. A preS mutation isolated from a patient with chronic hepatitis B infection leads to virus retention and misassembly. *Gastroenterology* 1997; **113**: 1976-1982 [PMID: 9394738]

115 **Bock CT**, Kubicka S, Manns MP, Trautwein C. Two control elements in the hepatitis B virus S-promoter are important for full promoter activity mediated by CCAAT-binding factor. *Hepatology* 1999; **29**: 1236-1247 [PMID: 10094970 DOI: 10.1002/hep.510290426]

116 **Bock CT**, Tillmann HL, Manns MP, Trautwein C. The pre-S region determines the intracellular localization and appearance of hepatitis B virus. *Hepatology* 1999; **30**: 517-525 [PMID: 10421662 DOI: 10.1002/hep.510300206]

117 **Ogata M**, Hino S, Saito A, Morikawa K, Kondo S, Kanemoto S, Murakami T, Taniguchi M, Tanii I, Yoshinaga K, Shiosaka S, Hammarback JA, Urano F, Imaizumi K. Autophagy is activated for cell survival after endoplasmic reticulum stress. *Mol Cell Biol* 2006;

26: 9220-9231 [PMID: 17030611 DOI: 10.1128/MCB.01453-06]

118   **Høyer-Hansen M**, Jäättelä M. Connecting endoplasmic reticulum stress to autophagy by unfolded protein response and calcium. *Cell Death Differ* 2007; **14**: 1576-1582 [PMID: 17612585 DOI: 10.1038/sj.cdd.4402200]

119   **Kouroku Y**, Fujita E, Tanida I, Ueno T, Isoai A, Kumagai H, Ogawa S, Kaufman RJ, Kominami E, Momoi T. ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. *Cell Death Differ* 2007; **14**: 230-239 [PMID: 16794605 DOI: 10.1038/sj.cdd.4401984]

120   **Li J**, Ni M, Lee B, Barron E, Hinton DR, Lee AS. The unfolded protein response regulator GRP78/BiP is required for endoplasmic reticulum integrity and stress-induced autophagy in mammalian cells. *Cell Death Differ* 2008; **15**: 1460-1471 [PMID: 18551133 DOI: 10.1038/cdd.2008.81]

121   **Qin L**, Wang Z, Tao L, Wang Y. ER stress negatively regulates AKT/TSC/mTOR pathway to enhance autophagy. *Autophagy* 2010; **6**: 239-247 [PMID: 20104019]

122   **Lépine S**, Allegood JC, Park M, Dent P, Milstien S, Spiegel S. Sphingosine-1-phosphate phosphohydrolase-1 regulates ER stress-induced autophagy. *Cell Death Differ* 2011; **18**: 350-361 [PMID: 20798685 DOI: 10.1038/cdd.2010.104]

123   **Sakaki K**, Wu J, Kaufman RJ. Protein kinase Ctheta is required for autophagy in response to stress in the endoplasmic reticulum. *J Biol Chem* 2008; **283**: 15370-15380 [PMID: 18356160 DOI: 10.1074/jbc.M710209200]

124   **Yorimitsu T**, Klionsky DJ. Endoplasmic reticulum stress: a new pathway to induce autophagy. *Autophagy* 2007; **3**: 160-162 [PMID: 17204854]

125   **Yorimitsu T**, Nair U, Yang Z, Klionsky DJ. Endoplasmic reticulum stress triggers autophagy. *J Biol Chem* 2006; **281**: 30299-30304 [PMID: 16901900 DOI: 10.1074/jbc.M607007200]

126   **Sir D**, Tian Y, Chen WL, Ann DK, Yen TS, Ou JH. The early autophagic pathway is activated by hepatitis B virus and required for viral DNA replication. *Proc Natl Acad Sci USA* 2010; **107**: 4383-4388 [PMID: 20142477 DOI: 10.1073/pnas.0911373107]

127   **Li J**, Liu Y, Wang Z, Liu K, Wang Y, Liu J, Ding H, Yuan Z. Subversion of cellular autophagy machinery by hepatitis B virus for viral envelopment. *J Virol* 2011; **85**: 6319-6333 [PMID: 21507968 DOI: 10.1128/JVI.02627-10]

128   **Naoumov NV**, Eddleston AL. Host immune response and variations in the virus genome: pathogenesis of liver damage caused by hepatitis B virus. *Gut* 1994; **35**: 1013-1017 [PMID: 7926896]

129   **Bertoletti A**, Ferrari C. Adaptive immunity in HBV infection. *J Hepatol* 2016; **64**: S71-S83 [PMID: 27084039 DOI: 10.1016/j.jhep.2016.01.026]

130   **Guidotti LG**, Chisari FV. Immunobiology and pathogenesis of viral hepatitis. *Annu Rev Pathol* 2006; **1**: 23-61 [PMID: 18039107 DOI: 10.1146/annurev.pathol.1.110304.100230]

131   **Peeridogaheh H**, Meshkat Z, Habibzadeh S, Arzanlou M, Shahi JM, Rostami S, Gerayli S, Teimourpour R. Current concepts on immunopathogenesis of hepatitis B virus infection. *Virus Res* 2018; **245**: 29-43 [PMID: 29273341 DOI: 10.1016/j.virusres.2017.12.007]

132   **Ando K**, Moriyama T, Guidotti LG, Wirth S, Schreiber RD, Schlicht HJ, Huang SN, Chisari FV. Mechanisms of class I restricted immunopathology. A transgenic mouse model of fulminant hepatitis. *J Exp Med* 1993; **178**: 1541-1554 [PMID: 8228807]

133   **Mina T**, Amini Bavil Olyaee S, Tacke F, Maes P, Van Ranst M, Pourkarim MR. Genomic Diversity of Hepatitis B Virus Infection Associated With Fulminant Hepatitis B Development. *Hepat Mon* 2015; **15**: e29477 [PMID: 26288637 DOI: 10.5812/hepatmon.29477v2]

134   **Bertoletti A**, Maini M, Williams R. Role of hepatitis B virus specific cytotoxic T cells in liver damage and viral control. *Antiviral Res* 2003; **60**: 61-66 [PMID: 14638399]

135   **Maini MK**, Boni C, Lee CK, Larrubia JR, Reignat S, Ogg GS, King AS, Herberg J, Gilson R, Alisa A, Williams R, Vergani D, Naoumov NV, Ferrari C, Bertoletti A. The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. *J Exp Med* 2000; **191**: 1269-1280 [PMID: 10770795]

136   **Bertoletti A**, Maini MK. Protection or damage: a dual role for the virus-specific cytotoxic T lymphocyte response in hepatitis B and C infection? *Curr Opin Immunol* 2000; **12**: 403-408 [PMID: 10899021]

137   **Bagaglio S**, Albarello L, Biswas P, Uberti-Foppa C, Fortis C, Morsica G. Virological pattern of hepatitis B infection in an HIV-positive man with fatal fulminant hepatitis B: a case report. *J Med Case Rep* 2009; **3**: 110 [PMID: 19946588 DOI: 10.1186/1752-1947-3-110]

138   **Raimondo G**, Allain JP, Brunetto MR, Buendia MA, Chen DS, Colombo M, Craxì A, Donato F, Ferrari C, Gaeta GB, Gerlich WH, Levrero M, Locarnini S, Michalak T, Mondelli MU, Pawlotsky JM, Pollicino T, Prati D, Puoti M, Samuel D, Shouval D, Smedile A, Squadrito G, Trépo C, Villa E, Will H, Zanetti AR, Zoulim F. Statements from the Taormina expert meeting on occult hepatitis B virus infection. *J Hepatol* 2008; **49**: 652-657 [PMID: 18715666 DOI: 10.1016/j.jhep.2008.07.014]

139   **Makvandi M**. Update on occult hepatitis B virus infection. *World J Gastroenterol* 2016; **22**: 8720-8734 [PMID: 27818588 DOI: 10.3748/wjg.v22.i39.8720]

140   **Chemin I**, Trépo C. Clinical impact of occult HBV infections. *J Clin Virol* 2005; **34** Suppl 1: S15-S21 [PMID: 16461218]

141   **Hashemi SJ**, Hajiani E, Masjedizadeh A, Makvandi M, Shayesteh AA, Alavinejad SP, Kadkhodaei A, Shahbazian H, Jasemi F, Karimi M. Occult hepatitis B infection in patients with cryptogenic liver cirrhosis in southwest of Iran. *Jundishapur J Microbiol* 2015; **8**: e16873 [PMID: 25861432 DOI: 10.5812/jjm.16873]

142   **Saitta C**, Tripodi G, Barbera A, Bertuccio A, Smedile A, Ciancio A, Raffa G, Sangiovanni A, Navarra G, Raimondo G, Pollicino T. Hepatitis B virus (HBV) DNA integration in patients with occult HBV infection and hepatocellular carcinoma. *Liver Int* 2015; **35**: 2311-2317 [PMID: 25677098 DOI: 10.1111/liv.12807]

143   **Chemin I**, Zoulim F, Merle P, Arkhis A, Chevallier M, Kay A, Cova L, Chevallier P, Mandrand B, Trépo C. High incidence of hepatitis B infections among chronic hepatitis cases of unknown aetiology. *J Hepatol* 2001; **34**: 447-454 [PMID: 11322208]

144   **Pollicino T**, Squadrito G, Cerenzia G, Cacciola I, Raffa G, Craxi A, Farinati F, Missale G, Smedile A, Tiribelli C, Villa E, Raimondo G. Hepatitis B virus maintains its pro-oncogenic properties in the case of occult HBV infection. *Gastroenterology* 2004; **126**: 102-110 [PMID: 14699492]

145   **Wong DK**, Huang FY, Lai CL, Poon RT, Seto WK, Fung J, Hung IF, Yuen MF. Occult hepatitis B infection and HBV replicative activity in patients with cryptogenic cause of hepatocellular carcinoma. *Hepatology* 2011; **54**: 829-836 [PMID: 21809355 DOI: 10.1002/hep.24551]

146   **Altfeld M**, Rockstroh JK, Addo M, Kupfer B, Pult I, Will H, Spengler U. Reactivation of hepatitis B in a long-term anti-HBs-positive patient with AIDS following lamivudine withdrawal. *J Hepatol* 1998; **29**: 306-309 [PMID: 9722213]

147   **Kidd-Ljunggren K**, Simonsen O. Reappearance of hepatitis B 10 years after kidney transplantation. *N Engl J Med* 1999; **341**: 127-128 [PMID: 10409028 DOI: 10.1056/NEJM199907083410213]

148   **Westhoff TH**, Jochimsen F, Schmittel A, Stoffler-Meilicke M, Schafer JH, Zidek W, Gerlich WH, Thiel E. Fatal hepatitis B virus reactivation by an escape mutant following rituximab therapy. *Blood* 2003; **102**: 1930 [PMID: 12930732 DOI: 10.1182/blood-2003-05-1403]

149   **Kubo S**, Tamori A, Ohba K, Shuto T, Yamamoto T, Tanaka H, Nishiguchi S, Wakasa K, Hirohashi K, Kinoshita H. Previous or occult hepatitis B virus infection in hepatitis C virus-associated hepatocellular carcinoma without hepatic fibrosis. *Dig Dis Sci* 2001; **46**: 2408-2414 [PMID: 11713944]

150   **Lalazar G**, Rund D, Shouval D. Screening, prevention and treatment of viral hepatitis B reactivation in patients with haematological malignancies. *Br J Haematol* 2007; **136**: 699-712 [PMID: 17338776 DOI: 10.1111/j.1365-2141.2006.06465.x]

151   **García-Fulgueiras A**, García-Pina R, Morant C, García-Ortuzar V,

Génova R, Alvarez E. Hepatitis C and hepatitis B-related mortality in Spain. *Eur J Gastroenterol Hepatol* 2009; **21**: 895-901 [PMID: 19357523 DOI: 10.1097/MEG.0b013e328313139d]

152 **Marcellin P**, Pequignot F, Delarocque-Astagneau E, Zarski JP, Ganne N, Hillon P, Antona D, Bovet M, Mechain M, Asselah T, Desenclos JC, Jougla E. Mortality related to chronic hepatitis B and chronic hepatitis C in France: evidence for the role of HIV coinfection and alcohol consumption. *J Hepatol* 2008; **48**: 200-207 [PMID: 18086507 DOI: 10.1016/j.jhep.2007.09.010]

153 **Xie M**, Rao W, Yang T, Deng Y, Zheng H, Pan C, Liu Y, Shen Z, Jia J. Occult hepatitis B virus infection predicts de novo hepatitis B infection in patients with alcoholic cirrhosis after liver transplantation. *Liver Int* 2015; **35**: 897-904 [PMID: 24750566 DOI: 10.1111/liv.12567]

154 **Candotti D**, Grabarczyk P, Ghiazza P, Roig R, Casamitjana N, Iudicone P, Schmidt M, Bird A, Crookes R, Brojer E, Miceli M, Amiri A, Li C, Allain JP. Characterization of occult hepatitis B virus from blood donors carrying genotype A2 or genotype D strains. *J Hepatol* 2008; **49**: 537-547 [PMID: 18602718 DOI: 10.1016/j.jhep.2008.04.017]

155 **Allain JP**, Belkhiri D, Vermeulen M, Crookes R, Cable R, Amiri A, Reddy R, Bird A, Candotti D. Characterization of occult hepatitis B virus strains in South African blood donors. *Hepatology* 2009; **49**: 1868-1876 [PMID: 19434719 DOI: 10.1002/hep.22879]

156 **Huang CH**, Yuan Q, Chen PJ, Zhang YL, Chen CR, Zheng QB, Yeh SH, Yu H, Xue Y, Chen YX, Liu PG, Ge SX, Zhang J, Xia NS. Influence of mutations in hepatitis B virus surface protein on viral antigenicity and phenotype in occult HBV strains from blood donors. *J Hepatol* 2012; **57**: 720-729 [PMID: 22634131 DOI: 10.1016/j.jhep.2012.05.009]

157 **Saha D**, Pal A, Sarkar N, Das D, Blackard JT, Guha SK, Saha B, Chakravarty R. Occult hepatitis B virus infection in HIV positive patients at a tertiary healthcare unit in eastern India. *PLoS One* 2017; **12**: e0179035 [PMID: 28591184 DOI: 10.1371/journal.pone.0179035]

158 **Ye Q**, Shang SQ, Li W. A new vaccine escape mutant of hepatitis B virus causes occult infection. *Hum Vaccin Immunother* 2015; **11**: 407-410 [PMID: 25692622 DOI: 10.4161/21645515.2014.994461]

159 **Hou J**, Wang Z, Cheng J, Lin Y, Lau GK, Sun J, Zhou F, Waters J, Karayiannis P, Luo K. Prevalence of naturally occurring surface gene variants of hepatitis B virus in nonimmunized surface antigen-negative Chinese carriers. *Hepatology* 2001; **34**: 1027-1034 [PMID: 11679975 DOI: 10.1053/jhep.2001.28708]

160 **Kwei K**, Tang X, Lok AS, Sureau C, Garcia T, Li J, Wands J, Tong S. Impaired virion secretion by hepatitis B virus immune escape mutants and its rescue by wild-type envelope proteins or a second-site mutation. *J Virol* 2013; **87**: 2352-2357 [PMID: 23221548 DOI: 10.1128/JVI.02701-12]

161 **Biswas S**, Candotti D, Allain JP. Specific amino acid substitutions in the S protein prevent its excretion in vitro and may contribute to occult hepatitis B virus infection. *J Virol* 2013; **87**: 7882-7892 [PMID: 23658444 DOI: 10.1128/JVI.00710-13]

162 **Zuckerman JN**, Zuckerman AJ. Mutations of the surface protein of hepatitis B virus. *Antiviral Res* 2003; **60**: 75-78 [PMID: 14638401]

163 **Xiang KH**, Michailidis E, Ding H, Peng YQ, Su MZ, Li Y, Liu XE, Dao Thi VL, Wu XF, Schneider WM, Rice CM, Zhuang H, Li T. Effects of amino acid substitutions in hepatitis B virus surface protein on virion secretion, antigenicity, HBsAg and viral DNA. *J Hepatol* 2017; **66**: 288-296 [PMID: 27650283 DOI: 10.1016/j.jhep.2016.09.005]

164 **Waters JA**, Kennedy M, Voet P, Hauser P, Petre J, Carman W, Thomas HC. Loss of the common "A" determinant of hepatitis B surface antigen by a vaccine-induced escape mutant. *J Clin Invest* 1992; **90**: 2543-2547 [PMID: 1281839 DOI: 10.1172/JCI116148]

165 **Shahmoradi S**, Yahyapour Y, Mahmoodi M, Alavian SM, Fazeli Z, Jazayeri SM. High prevalence of occult hepatitis B virus infection in children born to HBsAg-positive mothers despite prophylaxis with hepatitis B vaccination and HBIG. *J Hepatol* 2012; **57**: 515-521 [PMID: 22617152 DOI: 10.1016/j.jhep.2012.04.021]

166 **Jeantet D**, Chemin I, Mandrand B, Tran A, Zoulim F, Merle P, Trepo C, Kay A. Cloning and expression of surface antigens from occult chronic hepatitis B virus infections and their recognition by commercial detection assays. *J Med Virol* 2004; **73**: 508-515 [PMID: 15221893 DOI: 10.1002/jmv.20119]

167 **Minuk GY**, Sun DF, Uhanova J, Zhang M, Caouette S, Nicolle LE, Gutkin A, Doucette K, Martin B, Giulivi A. Occult hepatitis B virus infection in a North American community-based population. *J Hepatol* 2005; **42**: 480-485 [PMID: 15763333 DOI: 10.1016/j.jhep.2004.11.037]

168 **Wands JR**, Marciniak RA, Isselbacher KJ, Varghese M, Don G, Halliday JW, Powell LW. Demonstration of previously undetected hepatitis B viral determinants in an Australian Aboriginal population by monoclonal anti-hbs antibody radioimmunoassays. *Lancet* 1982; **1**: 977-980 [PMID: 6176820]

169 **Weinberger KM**, Bauer T, Böhm S, Jilg W. High genetic variability of the group-specific a-determinant of hepatitis B virus surface antigen (HBsAg) and the corresponding fragment of the viral polymerase in chronic virus carriers lacking detectable HBsAg in serum. *J Gen Virol* 2000; **81**: 1165-1174 [PMID: 10769057 DOI: 10.1099/0022-1317-81-5-1165]

170 **Zaaijer HL**, Torres P, Ontañón A, Ponte LG, Koppelman MH, Lelie PN, Hemert FJ, Boot HJ. Multiple surface antigen mutations in five blood donors with occult hepatitis B virus infection. *J Med Virol* 2008; **80**: 1344-1349 [PMID: 18551607 DOI: 10.1002/jmv.21233]

171 **Gish RG**, Yuen MF, Chan HL, Given BD, Lai CL, Locarnini SA, Lau JY, Wooddell CI, Schluep T, Lewis DL. Synthetic RNAi triggers and their use in chronic hepatitis B therapies with curative intent. *Antiviral Res* 2015; **121**: 97-108 [PMID: 26129970 DOI: 10.1016/j.antiviral.2015.06.019]

172 **Schluep T**, Lickliter J, Hamilton J, Lewis DL, Lai CL, Lau JY, Locarnini SA, Gish RG, Given BD. Safety, Tolerability, and Pharmacokinetics of ARC-520 Injection, an RNA Interference-Based Therapeutic for the Treatment of Chronic Hepatitis B Virus Infection, in Healthy Volunteers. *Clin Pharmacol Drug Dev* 2017; **6**: 350-362 [PMID: 27739230 DOI: 10.1002/cpdd.318]

173 **Zobeiri M**. Occult hepatitis B: clinical viewpoint and management. *Hepat Res Treat* 2013; **2013**: 259148 [PMID: 23533738 DOI: 10.1155/2013/259148]

REVIEW

Immunometabolism: A novel perspective of liver cancer microenvironment and its influence on tumor progression

Qi Zhang, Yu Lou, Xue-Li Bai, Ting-Bo Liang

Qi Zhang, Yu Lou, Xue-Li Bai, Ting-Bo Liang, Department of Hepatobiliary and Pancreatic Surgery, the Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310009, Zhejiang Province, China

Qi Zhang, Yu Lou, Xue-Li Bai, Ting-Bo Liang, Zhejiang Provincial Key Laboratory of Pancreatic Disease, Hangzhou 310009, Zhejiang Province, China

ORCID number: Qi Zhang (0000-0002-6096-0690); Yu Lou (0000-0003-2862-423X); Xue-Li Bai (0000-0001-6005-8384); Ting-Bo Liang (0000-0003-0143-3353).

Author contributions: Zhang Q and Lou Y contributed equally to this work; Liang TB and Bai XL designed the research; Zhang Q and Lou Y performed the research; Zhang Q and Lou Y wrote the paper; Liang TB and Bai XL revised the paper.

Conflict-of-interest statement: The authors declare that they have no competing interests.

Manuscript source: Invited manuscript

Correspondence to: Ting-Bo Liang, MD, PhD, Attending Doctor, Chief Doctor, Doctor, Full Professor, Research Fellow, Surgeon, Department of Hepatobiliary and Pancreatic Surgery, the Second Affiliated Hospital, Zhejiang University School of Medicine, No. 88 Jiefang Road, Hangzhou 310009, Zhejiang Province, China. liangtingbo@zju.edu.cn
Telephone: +86-571-87783510
Fax: +86-571-87783510

Abstract

The initiation and progression of liver cancer, including hepatocellular carcinoma and intrahepatic cholangiocarcinoma, are dependent on its tumor microenvironment. Immune cells are key players in the liver cancer microenvironment and show complicated crosstalk with cancer cells. Emerging evidence has shown that the functions of immune cells are closely related to cell metabolism. However, the effects of metabolic changes of immune cells on liver cancer progression are largely undefined. In this review, we summarize the recent findings of immunometabolism and relate these findings to liver cancer progression. We also explore the translation of the understanding of immunometabolism for clinical use.

Key words: Cholangiocarcinoma; Hepatocellular carcinoma; Tumor microenvironment; Local immune status; Metabolite

Core tip: The liver microenvironment provides a special place for initiation and progression of liver cancer, in which immune cells play a vital role. On the one hand, immunosuppression leads to tumor survival and progression; on the other hand, the instigation of tumor metabolites and signal molecules to immune cells makes the state of immunosuppression further strengthened. Intensive studies of the metabolic state of immune cells in the tumor microenvironment is beneficial to our understanding of the regulation of pro-tumor patterns, and to provide theoretical basis

and guidance for immunometabolic therapies for liver cancer.

INTRODUCTION

Liver cancer is one of the most common malignancies and has the third leading mortality rate and seventh leading incidence rate worldwide[1]. To date, with over 42000 cases diagnosed and 30000 deaths in the United States per year, a continuous increase in both morbidity and mortality is observed in liver cancer[2]. Unfortunately, the prognosis of advanced liver cancer is still poor despite the many treatments that have been developed in the past few decades. Thus, novel approaches to treat liver cancer are urgently needed.

The tumor microenvironment (TME) plays critical roles in tumor development and is characterized by complicated components, including various types of non-tumoral cells and non-cellular materials[3]. TMEs can be largely distinct among different types of cancers and among different patients with the same type of cancer. Within a patient, the TME can consistently change as the tumor progresses and is influenced by the physiopathological conditions of the patient. Thus, it is theoretically impossible to identify the precise state of the TME. However, under certain conditions, the TME can be specialized to show typical traits, and in particular, this specialized TME may affect tumor progression. Understanding these particular TMEs, if not all of them, can outline the crosstalk between the TME and tumor cells and facilitate the development of novel strategies for tumor treatment[4].

Pathologically, liver cancer mainly includes hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC). The two types of liver cancers share a similar hepatic microenvironment, but may have different TMEs due to the various biological characteristics of the tumor cells. For instance, in a considerable proportion of cases, ICC harbors isocitrate dehydrogenase (IDH) 1 and IDH2 mutations[5], which are rare in HCC[6]. IDH1 and IDH2 are important enzymes involved in cellular metabolism. Therefore, IDH1/2 mutations can substantially influence the metabolite profiles in cells and TMEs. In this case, the roles of the TME in liver cancer progression may be greatly altered. However, knowledge in this field is poor and scattered.

Immune cells are key players in liver cancer progression, and their recruitment and functions are profoundly affected by the TME[7]. Other molecules in the TME, such as fatty acids and glucose, are able to regulate the metabolism, phenotype and function of immune cells[8,9]. The term "immunometabolism" has recently been proposed and indicates the functional intracellular metabolic alterations that occur within immune cells[10]. These affected immune cells, in turn, could have significant effects on adjacent tumor cells. In this minireview, we collected evidence of TME heterogeneity resulting from the different biological features of cancer cells and functional changes of immune cells resulting from an altered TME and how the TME affects tumor progression *via* metabolically regulated immune cells.

CHARACTERISTICS OF IMMUNE CELLS IN LIVER CANCER MICROENVIRONMENT

With genetic and epigenetic changes, hepatoma cells express specific tumor-associated antigens, such as α-fetoprotein (AFP), glypican-3 (GPC3) and melanoma-associated gene-A1 (MAGE-A1), which can be taken up by antigen-presenting cells and presented to T cells, resulting in a cytotoxic reaction to eliminate cancerous cells[11,12]. However, immunosuppressive factors and immune-inhibitory checkpoint molecules inhibit anti-tumor reactions and create a special microenvironment to facilitate tumor progression[12]. Almost all types of immune cells are deeply involved in the TME of liver cancer (Figure 1), including macrophages, Kupffer cells, neutrophils, T cells, B cells, innate lymphoid cells (ILCs), dendritic cells (DCs), natural killer (NK) cells, natural killer T (NKT) cells, and myeloid-derived suppressor cells (MDSCs)[13-18].

Macrophages and neutrophils

Macrophages display remarkable heterogeneity in liver cancer for various reasons, such as the cell origin (resident Kupffer cells and recruited monocyte-derived macrophages), stimulating signals (*i.e.*, microbes, cell debris) and functional phenotype (*i.e.*, inflammatory, anti-inflammatory). Notably, macrophages can play two or more contradictory roles in a particular TME. As guardians, macrophages are normally activated by inflammatory signals and together with CD4+ T cells eliminate precancerous senescent cells[19]. Kupffer cells directly show cytotoxicity against tumor cells[20-22]. In addition, macrophage-derived interleukin (IL)-12 hampers tumor progression by activating NK and NKT cells[23,24]. As an accomplice, however, tumor-associated macrophages (TAMs, most of which are M2-polarized macrophages), induced by IL-4 and tumor growth factor (TGF)-β, accumulate in liver cancer and are correlated with the poor prognosis of patients[25,26]. Importantly, these macrophages express the immune checkpoint protein programmed cell death-ligand 1 (PD-L1, also known as B7-H1), which inactivates CD8+ T cells[13,27,28]. *Via* other immunosuppressive signals,

Figure 1 The immune cells in the tumor microenvironment regulate liver cancer progression. Many types of immune cells in the TME show pro- or anti-tumoral effects on the liver cancer cells by cell-specific mechanisms. Complicated crosstalk between immune cells is also common. TME: Tumor microenvironment; ILC: Innate lymphoid cell; NKT: Natural killer T.

such as Toll-like receptor (TLR) 4 and CD48/2B4, M2-polarized macrophages promote the recruitment of regulatory T cells (Tregs) and suppress the activity of NK cells[29-31]. Moreover, these macrophages can secrete various tumor proliferation-promoting cytokines, such as IL-1β, IL-6, TGF-β, C-X-C motif chemokine (CXCL) 10, invasion and metastasis-promoting factors like tumor necrosis factor (TNF)-α, osteopontin (OPN), matrix metalloproteinases (MMPs), C-C Motif chemokine ligand (CCL) 22, and proangiogenic growth factors, like vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), and TGF-β, to build a tumor-prone inflammatory microenvironment[3,26,32-35].

Similar to macrophages, neutrophils also have diverse functions at different stages of liver cancer progression. In the case of a hepatic infection or injury, neutrophils gather at the wound site together with macrophages to eliminate pathogens and necrotic materials. Additionally, neutrophils stimulate reactive oxygen species (ROS) and telomere DNA damage in hepatocytes, mediating neoplasia and progression[36]. Mirroring macrophage plasticity, a pro-tumoral phenotype of tumor-associated neutrophils (TANs) is proposed[37,38]. Despite biomarkers of this subtype, immunosuppression is the most central function of TANs. The immunosuppressive molecule PD-L1 is regularly displayed in TANs[39] and recruits macrophages and Treg cells to the liver cancer TME and induces impaired anti-tumoral immunity[14]. The infiltrating TAN density and neutrophil-lymphocyte ratio is reported to be a predictor of outcome, chemotherapy resistance, and recurrence risk[40-42]. Furthermore, neutrophils promote tumor progression by secreting cytokines and other functional molecules, such as CCL2

for tumor growth, hepatocyte growth factor (HGF) and oncostatin M (OSM) for metastasis, and MMP9 and VEGF for angiogenesis[38,43-47].

T cells

CD8^{+} T cells are the most important executors of adaptive immunity against neoplasms, including liver cancer. Unfortunately, the TME transforms these 'warriors' into 'servants'. Compared with the normal liver, tumor tissue has a lower density of CD8^{+} T cells and a higher density of Tregs. The ratio of CD8^{+} T cells to Tregs typically indicates a poor prognosis[48-50]. Recent studies suggest that interferon (IFN)-γ, TNF and granzyme secretion by CD8^{+} cytotoxic T lymphocytes (CTLs) represent a common cytotoxic reaction against tumors[51,52]. Tregs, characterized by CD4, CD25, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and forkhead box P3 (FoxP3) expression, can eliminate IL-2 *via* its receptor subunit CD25, downregulate CD80 and CD86 and conjugate to the co-stimulatory molecule CD28 competitively with CTLA-4 to suppress immune responses. In addition, Tregs secrete TGF-β and IL-10 into the TME to suppress T effector cells[52]. *Via* a complicated regulatory network, several subtypes of T cells contribute to the immunosuppressive TME.

ILCs

ILCs are recently identified innate immune cells that lack a specific antigen receptor. These cells originate from mucosal-associated lymphoid tissues and act as a sentry of the rapid immune response and regulator of immune homeostasis and inflammation[53]. Mirroring the classification of helper T cells (Th), ILCs are divided into three classes. ILC1s produce Th1-associated cytokines, ILC2s are associated with Th2-associated cytokine release, and ILC3s secrete Th17-associated cytokines[54]. As these three components simultaneously exist in the liver, they are likely to be involved in hepatocarcinogenesis and progression. ILC1s produce IFN-γ to activate NK cells and indirectly participate in cancer immunosurveillance, while ILC3s release IL-17 and IL-22, which may promote tumor growth and angiogenesis[16,55,56]. Moreover, we observed an increasing number of ILC2 in HCC tissue (unpublished data) and an elevated level of IL-33 in both serum and tumor tissue samples from HCC patients[57]. IL-33 can stimulate rapid growth and metastatic progression in breast cancer and cholangiocarcinoma[58,59]. Therefore, the initiation and growth of liver cancer promoted by the ILC2-related IL-33/ST2 axis might be theoretically tenable[60].

ALTERED METABOLISM OF LIVER CANCER AND ITS INFLUENCE ON THE TUMOR MICROENVIRONMENT

With their rapid growth and limited nutrition supply,

the survival of tumor cells seems to be in question. However, the tumor is able to reprogram the cellular metabolism for neoplastic proliferation. The abnormal metabolism induced by cancer provides energy and metabolites for cell activity and additionally modifies many related pathways that influence various biological processes[61].

General alterations

The liver is a metabolic organ, and metabolic disruption of the liver can lead to spontaneous hepatocarcinogenesis[62]. The principal metabolic alterations in the liver cancer were profiled by metabolomics analysis, which showed elevated glycolysis, gluconeogenesis and β-oxidation, with reduced tricarboxylic acid cycle (TCA cycle) activity[63,64]. Aerobic glycolysis, also known as the "Warburg effect", is frequently observed in various tumors. This theory indicates that tumor cells predominantly use glycolysis, even in the presence of sufficient oxygen[65]. For liver cancer, glycolysis also plays a dominating role in glucose metabolism[63]. Accumulating studies have revealed that glycolysis is associated with genetic and epigenetic changes. Activated oncogenes and mutant tumor suppressors are associated with glycolysis. Glucose transporters (GLUTs), glycolysis-related enzyme hexokinase2 (HK2), pyruvate kinase M2 (PKM2), and lactate dehydrogenase (LDH) A are also overexpressed in liver cancer tissue, suggesting an increased glycolysis activity[66]. Moreover, the hypoxic TME and overexpression of β-catenin in liver cancer stabilize hypoxia inducible factor (HIF)-1α to activate glycolytic enzymes[67-70].

Fatty acid β-oxidation is enhanced in liver cancer to overcome the energy shortage and reduce tumor dependence on glucose. Metabolic alteration increases FA biosynthesis and glycerolipid metabolism, leading to fatty acid and lipid accumulation[71,72]. However, the alterations of various types of fatty acids are complicated. Additionally, almost all amino acids are increased in liver cancer due to the reduction of amino acid catabolism[63]. Although these general changes were identified, many other factors may influence the levels of metabolites in TME.

Involvement of specific mutations

Some specific mutations profoundly change the metabolism of liver cancer. The Tumor Cell Genomic Atlas (TCGA) project of HCC demonstrated that IDH and fibroblast growth factor receptor (FGFR) are meaningful mutations in HCC. IDHs are critical enzymes for cell metabolism, particularly the TCA cycle. IDH1/2 mutations convert α-ketogluterate (α-KG) into 2-hydrogluterate (2-HG)[73]. The unusually decreased ratio of α-KG/2-HG can significantly influence tumor cell biology by competing with α-KG and regulating epigenetic expression[74]. However, overproduced 2-HG by tumor cells can also be released into the TME since elevated serum and urinary levels of 2-HG can

be detected in patients with IDH1/2-mutated solid tumors[75,76]. Although 2-HG seems to be impermeable, this enzyme acts on stromal cells within the bone marrow microenvironment through an ROS/extracellular signal-regulated kinase (ERK) signaling pathway[77], suggesting that 2-HG may influence immune cells in the TME of liver cancer. This finding is important since IDH-like HCCs (particularly those containing IDH1/2 mutations and those with IDH-like gene expression) are indicative of worse clinical outcomes for undefined reasons[6]. In addition to 2-HG, IDH-like HCCs and IDH1/2 mutated ICCs have other significant metabolic alterations, such as gliomas due to pseudohypoxia, an interrupted Krebs cycle, and epigenetic changes[78]. In addition, IDH gain-of-function mutations also induce the reprogramming of pyruvate and lipid metabolism to maintain cell proliferation and clonogenicity[79,80]. Therefore, the TMEs of these tumors likely have distinct features, and the immune cells within such TMEs may be reprogrammed to have different functions.

FGFR genetic aberrations include mutations, amplifications, and gene fusions, which have been observed in over 10% of liver cancer[81-83]. FGFR2 fusions are active kinases with the highest incidence[84]. These genetic aberrations indirectly affect glucose and lipid metabolism by activating the kinase network[85]. Additionally, the oncogenic FGFR3-transforming acidic coiled-coil containing protein (TACC) 3 fusion activates oxidative phosphorylation and mitochondrial biogenesis, causing a mitochondrial respiration-dependent subtype of tumor cell[86].

Influence of hepatic viruses

Hepatitis viruses, particularly HBV in eastern countries and HCV in western countries, cause specific metabolic alterations during hepatocarcinogenesis and tumor progression. Hepatic virus infections activate many abnormal signaling pathways, which cause aberrant functions and expression of metabolism related enzymes[87]. As a consequence, HBV infection in patients is associated with increased free fatty acids (FFAs) and acyl-carnitines and decreased triglycerides, phospholipids, and sphingomyelins[88]. Thus, in the TME of HBV-related liver cancer, the increased FFAs and decreased glucose may result from increased β-oxidation and glycolysis, respectively. HBV-related inflammation can stimulate the expression of HIF-1α through the PI3K/AKT and mitogen-activated protein kinase (MAPK)-Ras-Raf pathways. The latter up-regulates GLUT1 and glycolytic enzymes, increases the glycolytic flow, and induces ROS accumulation. These alterations eventually cause DNA oxidative damage and malignant transformation[89-92]. HBx activates the adenosine 5'-monophosphate-activated protein kinase (AMPK) and fatty acid oxidation (FAO) pathways as well as the HBx-LXRα-SREBP1/FAS pathway, which allows HCC cells to survive under metabolic stress[93-95]. HCV establishes an insulin-resistant TME in the liver through several mechanisms[96,97]. For instance, HCV protein can induce the overexpression of protein phosphatase 2A (PP2A), which dysregulates hepatic glucose homeostasis by inhibiting AKT and the dephosphorylation of FoxO1[98]. However, the molecular mechanism of viral hepatitis-related metabolic alternations is not well understood. HBV integration into the tumor suppressor gene region causes metabolic alterations. Signaling molecules, such as TGF-β, mammalian target of rapamycin (mTOR), Smad3, and c-Myc, are activated by HBV and HCV, which is closely correlated with tumor progression[87,96].

Participation of other concomitant diseases

Liver cancer has a higher incidence in people with chronic non-infectious liver diseases. Therefore, the metabolic changes of liver cancer are related to cirrhosis and nonalcoholic fatty liver disease (NAFLD), which typically includes lipid anomalies, particularly dysregulated *de novo* lipogenesis[99]. For instance, peripheral insulin resistance together with enhanced mitochondrial β-oxidation and oxidative stress are the most prominent features of NAFLD and nonalcoholic steatohepatitis (NASH)[100]. Thus, NAFLD-related liver cancer is associated with elevated oxidative metabolism and amplified anaplerosis/cataplerosis[101]. With the increasing content of hepatic FFAs, liver cells may endure a mild respiratory dysfunction. The increasing import of FFAs into the mitochondria is accompanied by an elevated rate of β-oxidation. The overloaded fatty acid β-oxidation triggers the subsequent accumulation of ROS, which further leads to lipid peroxidation and severe mitochondrial oxidative damage[102,103]. These strong oxidizing products promote inflammation, fibrosis, and even carcinogenesis. Lipid metabolism shows an alteration from β-oxidation to ω-oxidation during liver cirrhosis[104]. Intriguingly, a metabolic switch from oxidative phosphorylation to glycolysis in hepatocytes in early-stage cirrhosis may satisfy the extreme energy requirements under such conditions[105]. This evidence demonstrates that concomitant liver diseases or even systemic metabolic diseases, such as diabetes mellitus, can influence the function of immune cells and their functions in promoting tumor progression.

Metabolic reprogramming of local immune cells in liver cancer

The role of metabolism in regulating immune cells has recently aroused general concerns. Evidence collected in several types of solid tumors indicated the importance of tumor immunometabolic reprogramming and suggested a novel and crucial area for future research of liver cancer[106]. The complicated crosstalk between metabolically reprogrammed immune cells and liver cancer cells has been suggested, but the molecular mechanisms need further exploration (Figure 2).

Because they exist at a considerable quantity, macrophages play a leading role in the crosstalk between liver cancer cells and immune cells. The
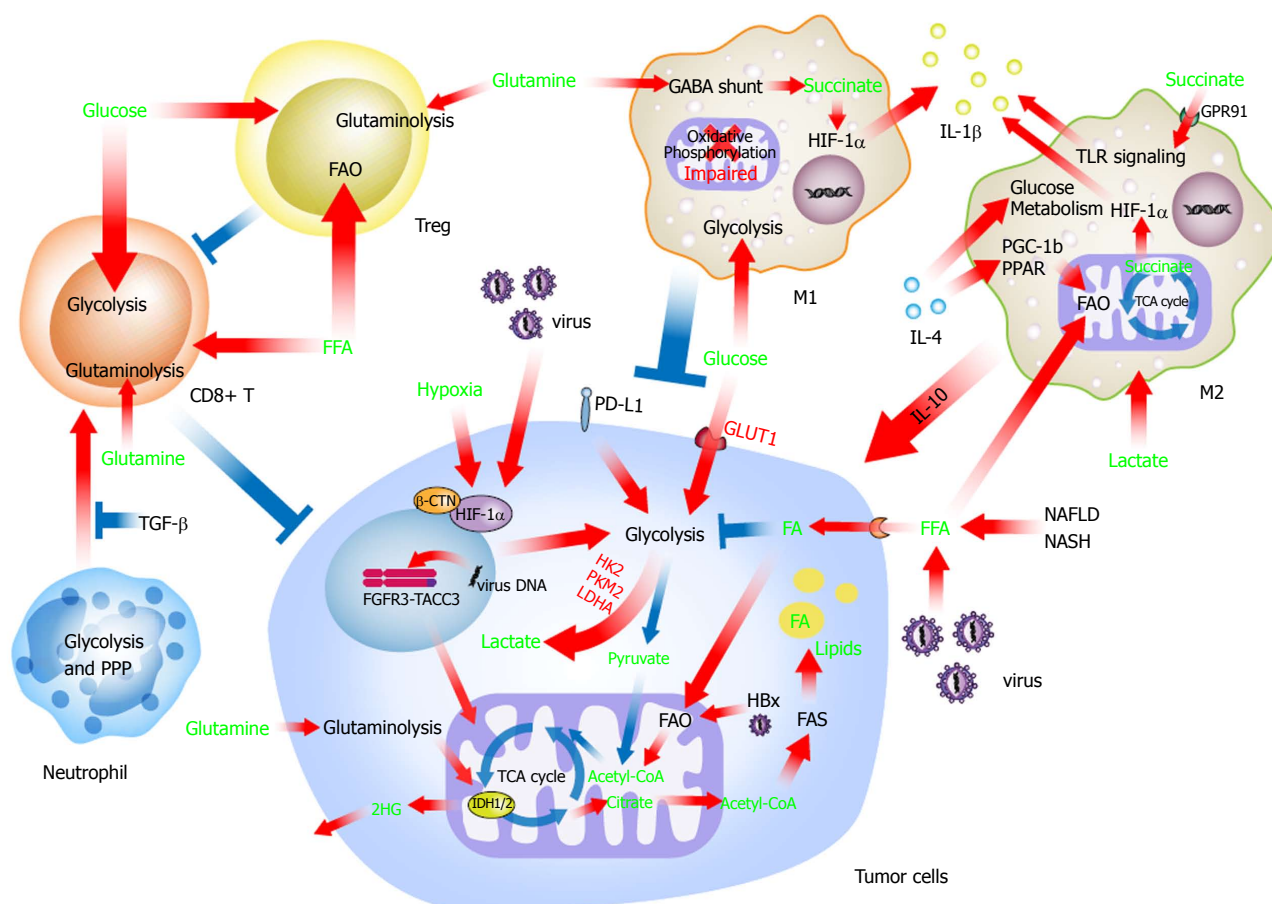
Figure 2 Metabolites in the tumor microenvironment affect the anti-/pro-tumoral functions of immune cells. Energy resources including glucose, glutamine, free fatty acids, and other metabolites such as succinate largely alter the functions of macrophages, neutrophils, and T cells. These metabolically reprogrammed immune cells then have a differed influence on the liver cancer cells compared to the original immune cells. FAO: Fatty acid oxidation.

phenotypes and functions of macrophages are hot topics in the field of immunometabolism. Professor O'Neill's group and other investigations have conducted several pioneering and important studies to decipher macrophage immunometabolism. Recent evidence has suggested that the energy source (*e.g.*, glucose and fatty acids) participates in the determination of macrophage polarization[107,108]. However, the role of FAO in the alternative activation of macrophages is still under debate. Huang *et al*[108] initially demonstrated an essential role for FAO in the alternative activation of mouse macrophages, while two groups in Germany and the United States subsequently provided convincing evidence that FAO was indispensable for the M2 polarization of human and mouse macrophages, respectively[109,110]. This discrepancy may partially reflect differences between mouse and human macrophages and the potential off-target effects of etomoxir (a carnitine palmitoyltransferase I A, CPT1A inhibitor used to inhibit FAO by some researchers). Although the role of FAO in macrophage activation has not yet been determined, FAO enhances the functions of M2 polarized macrophages[111].

Generally, M2 polarized macrophages use oxidative phosphorylation, FAO in particular, to obtain the required energy and exert pro-tumor function in various scenarios, including liver cancer[108,112,113]. Mechanistically, IL-4 induces the expression of peroxisome proliferator-activated receptors (PPAR) and PPAR gamma coactivator 1-beta (PGC-1b), which orchestrate alteration of FAO as well as mitochondrial respiration[114]. Similar to liver cancer cells in a background of chronic fatty liver disease, FFA accumulation attenuates mitochondrial respiration and increases FFA uptake *via* CD36, resulting in a predominant FAO mode in macrophages[115,116]. In addition, IL-4 signaling also increases glucose metabolism by the AKT/mTOR pathway[117-119]. Lactic acid produced by liver cancer cells through glycolysis is another non-negligible issue when considering the metabolic reprogramming of liver cancer-associated macrophages. The polarization of macrophages to an immunosuppressive and pro-tumoral phenotype is mediated by lactic acid *via* a different, as yet unidentified pathway[119-121] in liver cancer. M2 polarized macrophages also take up insulin growth factor 1 for a self-immunometabolic reprogramming, leading to reduced phagocytosis and lower energy expenditure[122], which may consequently regulate liver cancer progression.

However, M1 polarized macrophages have enhanced glycolytic metabolism and impaired oxidative phosph-

orylation through the AKT/mTOR/HIF-1α pathway[123]. This association is consistent with the fact that many glycolytic enzymes facilitate inflammatory cytokine production. For example, PKM2, which is critical for glycolysis, activates HIF-1α and induces IL-1β production[124]. Although under some conditions, FAO was also found to support inflammasome activation in M1 polarized macrophages by regulating CPT1A activity[125,126].

Other metabolites in the TME can also influence the phenotype and function of macrophages. Succinate is a known inflammation inducer of macrophages. Succinate enhances IL-1β secretion *via* succinate receptor 1 (SUCNR1, also known as GPR91)-mediated amplification of TLR signaling[127]. In addition, intracellular succinate, derived from the γ-aminobutyric acid (GABA) shunt and anerplerosis of α-KG upon stimulation of TLR4, enhances IL-1β production through the repurposing of mitochondrial function from ATP production to ROS generation[128,129].

Neutrophils are metabolically similar to M1 polarized macrophages and rely on aerobic glycolysis and the pentose phosphate pathway (PPP) as their principal mode of energy metabolism, by which the formation of neutrophil extracellular traps produces biological effects[130].

During differentiation, a switch from FAO to glycolysis and glutaminolysis triggers the maturation of T effector cells[131]. The rapid supply of ATP helps CTLs to meet their increasing bioenergetic and biosynthetic requirements[132]. Nevertheless, glucose shortage and lactic acid abundance limit the function of CTLs, and the enhancement of FAO preserves the cytotoxicity of CTLs in tumors[133]. By contrast, Treg cells favor FAO rather than glycolysis, by which these cells survive in the persistent low-glucose and hypoxic tumor microenvironment and suppress the tumor-killing function of CTLs[131,134]. Moreover, Treg cells rely on oxidative phosphorylation for energy supply, on FAO for cell differentiation, and on glutaminolysis for cell proliferation[134,135]. Taken together, these observations demonstrate that environment-related metabolism regulates the anti- and pro-tumor functions of tumor-infiltrating lymphocytes.

INFLUENCE OF REPROGRAMMED IMMUNE CELLS ON LIVER CANCER PROGRESSION

Immunometabolic reprogramming has a dual-function in tumor progression. Typically, M1 polarized macrophages facilitate inflammation and the antitumor response *via* elevated glycolysis, while M2 polarized macrophages play an FAO predominant role, secreting IL-10 to suppress the immune reaction. The switch in the metabolism of TAMs leads to an ample signaling transition by which these cells suppress immune reactions (by presenting PD-1, *etc.*), accelerate tumor proliferation (by releasing TNF, Wnt signals, *etc.*), promote angiogenesis (by secreting VEGF, FGF2, *etc.*), and enhance tumor invasion and metastasis (by MMP9, TGF-β, *etc.*)[26,136]. We further revealed that IL-1β facilitated epithelial-to-mesenchymal transition and subsequent metastasis in liver cancer, and this effect was mediated by a group of pro-inflammatory M2-like TAMs with an up-regulated level of glycolysis[137]. Interestingly, the function of TANs is opposite that of TAMs under different circumstances. For instance, the absence of TGF-β leads to a TANs-induced anti-tumor response by CTLs, whereas this situation is completely different in the presence of TGF-β[37].

The influence of metabolic reprogramming on lymphocytes differs in the presence of high functional heterogeneity. CTLs enhance glycolysis, glutaminolysis, and even FAO to exert anti-tumoral cytotoxicity, while CD4+ T cells develop into two phenotypes with contrary functions. With a similar switch characterized by up-regulated glycolysis as well as increased glutaminolysis and PPP, Th1 cells induce macrophage- and NK cell-related anti-tumoral responses, whereas Th2 cells induce immunosuppressive reactions[113].

PERSPECTIVES OF LIVER CANCER THERAPY FROM IMMUNOMETABOLISM

Theoretically, it is feasible to target metabolic enzymes or metabolites in immune cells for therapeutic purposes. Indeed, several cancer-related immunometabolic molecules are suitable as potential targets for drug development (Table 1).

Considering carbohydrate metabolism, glycolysis is important to activated immune cells. Inhibition of HIF-1α can attenuate TAM/TAN-mediated IL-1β secretion, reduce hypoxic adaptation of tumor cells, and regulate the differentiation and function of lymphocytes[137,138]. The regulation of PI3K signaling by PTEN in immune cells may also show some effects on macrophage activation and Treg cell function with a concurrent influence on glycolysis[139,140].

The mTOR pathway may be a suitable target to regulate immune cells by manipulating cellular lipid metabolism. Inhibition of mTOR by rapamycin can block the development of macrophages and CD4+ T cells in several scenarios, including liver cancer[141,142]. Moreover, FAO plays a predominant role in the metabolism of M2 polarized macrophages. Hence, inhibition of FAO by etomoxir or other methods (*e.g.*, targeted delivery of *CPT1A* siRNA/shRNA) may limit the immunosuppressive function of M2 macrophages. However, in a resource-deficient microenvironment, CTLs also take up FFA and exert cytotoxicity; thus, the selective increase of FFA import to CTLs enhances the anti-tumoral capacity of these cells[133]. It is also feasible to target proteins that play central roles in metabolic pathways (*i.e.*, iNOS, PKM2, Foxp3, *etc.*). The difficulty of this strategy is that targeting should be cell specific because the same metabolic pathway can have diverse effects on different

Table 1 Immunometabolic therapies for cancers

Targets	Agents	Mechanisms	Developments
PKM2	DASA, TEPP46	Inhibition of HIF1α	Preclinical
HIF1α	PX-478, RO7070179, EZN-2968	Inhibition of HIF1α	Phase 1
PTEN	VO-Ohpic, SF1670	Inhibition of PI3K	Preclinical
PDK	Dichloroacetate	Inhibition of glycolysis	Phase 2
GLUT1	Ritonavir	Inhibition of glycolysis	FDA-Approved
LDH	Gossypol (AT-101), FX11, Galloflavin	Inhibition of glycolysis	Phase 3
CPT1A	Etomoxir	Inhibition of FAO	Preclinical
CTLA-4	Ipilimumab	Checkpoint blockade; Inhibition of FAO	FDA-Approved
PD-1/PD-L1	Nivolumab, Pembrolizumab, Atezolizumab	Checkpoint blockade; Inhibition of FAO	FDA-Approved
AMPK	Metformin	Increased FAO; Inhibition of Complex I; Decreased mitochondrial ROS	FDA-Approved
mTOR	Temsirolimus, Everolimus	Inhibition of HIF-1α translation	FDA-Approved
IDO	Epacadostat	Regulation of tryptophan metabolism; Inhibition of mTORC1	FDA-Approved
IDH1/2 mutations	Ivosidenib (AG-120), IDH305, AG-881, DS-1001b	Inhibition of 2-HG production	Phase 3
FGFR	Regorafenib, Sunitinib, TAS120	Inhibition multi-targeted kinase	Phase 2
iNOS	L-NMMA, 1400W	Inhibition of NO production	Phase 2
FOXP3	P60	suppress NF-κB and NFAT	Preclinical

PKM2: Pyruvate kinase M2; HIF: Hypoxia inducible factor; LDH: Lactate dehydrogenase; FAO: Fatty acid oxidation; CTLA-4: Cytotoxic T-lymphocyte-associated protein 4; mTOR: Mammalian target of rapamycin; FGFR: Fibroblast growth factor receptor; FoxP3: Forkhead box P3; ROS: Reactive oxygen species.

immune cells.

Furthermore, drugs targeting tumor specific metabolic changes can also regulate the function of immune cells by altering the metabolites of the tumor. For instance, IDH1/2 inhibitors specifically reduce 2-HG production leading to destabilization of HIF-1α in immune cells, which can cause reduction of IL-1β secretion by macrophages.

CONCLUSION

Immunometabolism has increasingly become a component of immunology in the past decade. The current understanding suggests that a complicated metabolic network regulates the various functions of immune cells. This network can either be functionally oriented or environmentally adapted, but together, these complex interactions lead to immune microenvironment homeostasis, which affects the progression of liver cancer. However, numerous findings indicate that almost all types of immune cells present a self-contradictory function under different conditions, although the mechanisms are still unknown. In the future, more subtypes among immune cells will be defined as single cell detection systems are further developed, which may provide a key for functional heterogeneity. Therefore, liver cancer therapy that targets immunometabolism is a promising approach. Further work is urgently needed to explore the utility of targeting specific immunometabolic events in the liver cancer microenvironment for therapeutic gain.

REFERENCES

1   Fact sheets by Population-Globocan-IARC. IARC 2012
2   **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* 2018; **68**: 7-30 [PMID: 29313949 DOI: 10.3322/caac.21442]
3   **Quail DF**, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nat Med* 2013; **19**: 1423-1437 [PMID: 24202395 DOI: 10.1038/nm.3394]
4   **Fang H**, Declerck YA. Targeting the tumor microenvironment: from understanding pathways to effective clinical trials. *Cancer Res* 2013; **73**: 4965-4977 [PMID: 23913938 DOI: 10.1158/0008-5472.CAN-13-0661]
5   **Farshidfar F**, Zheng S, Gingras MC, Newton Y, Shih J, Robertson AG, Hinoue T, Hoadley KA, Gibb EA, Roszik J, Covington KR, Wu CC, Shinbrot E, Stransky N, Hegde A, Yang JD, Reznik E, Sadeghi S, Pedamallu CS, Ojesina AI, Hess JM, Auman JT, Rhie SK, Bowlby R, Borad MJ; Cancer Genome Atlas Network, Zhu AX, Stuart JM, Sander C, Akbani R, Cherniack AD, Deshpande V, Mounajjed T, Foo WC, Torbenson MS, Kleiner DE, Laird PW, Wheeler DA, McRee AJ, Bathe OF, Andersen JB, Bardeesy N, Roberts LR, Kwong LN. Integrative Genomic Analysis of Cholangiocarcinoma Identifies Distinct IDH-Mutant Molecular Profiles. *Cell Rep* 2017; **18**: 2780-2794 [PMID: 28297679 DOI: 10.1016/j.celrep.2017.02.033]
6   **Cancer Genome Atlas Research Network.** Cancer Genome Atlas Research Network. Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma. *Cell* 2017; **169**: 1327-1341.e23 [PMID: 28622513 DOI: 10.1016/j.cell.2017.05.046]
7   **Gajewski TF**, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. *Nat Immunol* 2013; **14**: 1014-1022 [PMID: 24048123 DOI: 10.1038/ni.2703]
8   **Yaqoob P**. Fatty acids as gatekeepers of immune cell regulation. *Trends Immunol* 2003; **24**: 639-645 [PMID: 14644137]
9   **Lawless SJ**, Kedia-Mehta N, Walls JF, McGarrigle R, Convery O, Sinclair LV, Navarro MN, Murray J, Finlay DK. Glucose represses dendritic cell-induced T cell responses. *Nat Commun* 2017; **8**: 15620 [PMID: 28555668 DOI: 10.1038/ncomms15620]
10   **O'Neill LA**, Kishton RJ, Rathmell J. A guide to immunometabolism for immunologists. *Nat Rev Immunol* 2016; **16**: 553-565 [PMID: 27396447 DOI: 10.1038/nri.2016.70]
11   **Flecken T**, Schmidt N, Hild S, Gostick E, Drognitz O, Zeiser R, Schemmer P, Bruns H, Eiermann T, Price DA, Blum HE, Neumann-Haefelin C, Thimme R. Immunodominance and functional alterations of tumor-associated antigen-specific CD8+

T-cell responses in hepatocellular carcinoma. *Hepatology* 2014; **59**: 1415-1426 [PMID: 24002931 DOI: 10.1002/hep.26731]

12   **Prieto J**, Melero I, Sangro B. Immunological landscape and immunotherapy of hepatocellular carcinoma. *Nat Rev Gastroenterol Hepatol* 2015; **12**: 681-700 [PMID: 26484443 DOI: 10.1038/nrgastro.2015.173]

13   **Wu K**, Kryczek I, Chen L, Zou W, Welling TH. Kupffer cell suppression of CD8+ T cells in human hepatocellular carcinoma is mediated by B7-H1/programmed death-1 interactions. *Cancer Res* 2009; **69**: 8067-8075 [PMID: 19826049 DOI: 10.1158/0008-5472.can-09-0901]

14   **Zhou SL**, Zhou ZJ, Hu ZQ, Huang XW, Wang Z, Chen EB, Fan J, Cao Y, Dai Z, Zhou J. Tumor-Associated Neutrophils Recruit Macrophages and T-Regulatory Cells to Promote Progression of Hepatocellular Carcinoma and Resistance to Sorafenib. *Gastroenterology* 2016; **150**: 1646-1658.e17 [PMID: 26924089 DOI: 10.1053/j.gastro.2016.02.040]

15   **Shi JY**, Gao Q, Wang ZC, Zhou J, Wang XY, Min ZH, Shi YH, Shi GM, Ding ZB, Ke AW, Dai Z, Qiu SJ, Song K, Fan J. Margin-infiltrating CD20(+) B cells display an atypical memory phenotype and correlate with favorable prognosis in hepatocellular carcinoma. *Clin Cancer Res* 2013; **19**: 5994-6005 [PMID: 24056784 DOI: 10.1158/1078-0432.ccr-12-3497]

16   **Dadi S**, Chhangawala S, Whitlock BM, Franklin RA, Luo CT, Oh SA, Toure A, Pritykin Y, Huse M, Leslie CS, Li MO. Cancer Immunosurveillance by Tissue-Resident Innate Lymphoid Cells and Innate-like T Cells. *Cell* 2016; **164**: 365-377 [PMID: 26806130 DOI: 10.1016/j.cell.2016.01.002]

17   **Gonzalez-Carmona MA**, Lukacs-Kornek V, Timmerman A, Shabani S, Kornek M, Vogt A, Yildiz Y, Sievers E, Schmidt-Wolf IG, Caselmann WH, Sauerbruch T, Schmitz V. CD40ligand-expressing dendritic cells induce regression of hepatocellular carcinoma by activating innate and acquired immunity in vivo. *Hepatology* 2008; **48**: 157-168 [PMID: 18537185 DOI: 10.1002/hep.22296]

18   **Schrader J**. The role of MDSCs in hepatocellular carcinoma--in vivo veritas? *J Hepatol* 2013; **59**: 921-923 [PMID: 23958935 DOI: 10.1016/j.jhep.2013.08.003]

19   **Eggert T**, Wolter K, Ji J, Ma C, Yevsa T, Klotz S, Medina-Echeverz J, Longerich T, Forgues M, Reisinger F, Heikenwalder M, Wang XW, Zender L, Greten TF. Distinct Functions of Senescence-Associated Immune Responses in Liver Tumor Surveillance and Tumor Progression. *Cancer Cell* 2016; **30**: 533-547 [PMID: 27728804 DOI: 10.1016/j.ccell.2016.09.003]

20   **Aono K**, Isobe K, Nakashima I, Kondo S, Miyachi M, Nimura Y. Kupffer cells cytotoxicity against hepatoma cells is related to nitric oxide. *Biochem Biophys Res Commun* 1994; **201**: 1175-1181 [PMID: 8024559 DOI: 10.1006/bbrc.1994.1829]

21   **Saito H**, Kurose I, Ebinuma H, Fukumura D, Higuchi H, Atsukawa K, Tada S, Kimura H, Yonei Y, Masuda T, Miura S, Ishii H. Kupffer cell-mediated cytotoxicity against hepatoma cells occurs through production of nitric oxide and adhesion via ICAM-1/CD18. *Int Immunol* 1996; **8**: 1165-1172 [PMID: 8757962]

22   **Li XY**, Wu L, Li SW, Zhou WB, Wang MY, Zuo GQ, Liu CA, Ding X. Effect of CD16a, the surface receptor of Kupffer cells, on the growth of hepatocellular carcinoma cells. *Int J Mol Med* 2016; **37**: 1465-1474 [PMID: 27082928 DOI: 10.3892/ijmm.2016.2561]

23   **Rushfeldt C**, Sveinbjørnsson B, Seljelid R, Smedsrød B. Early events of hepatic metastasis formation in mice: role of Kupffer and NK-cells in natural and interferon-gamma-stimulated defense. *J Surg Res* 1999; **82**: 209-215 [PMID: 10090831 DOI: 10.1006/jsre.1998.5532]

24   **Seki S**, Nakashima H, Nakashima M, Kinoshita M. Antitumor immunity produced by the liver Kupffer cells, NK cells, NKT cells, and CD8 CD122 T cells. *Clin Dev Immunol* 2011; **2011**: 868345 [PMID: 22190974 DOI: 10.1155/2011/868345]

25   **Ding T**, Xu J, Wang F, Shi M, Zhang Y, Li SP, Zheng L. High tumor-infiltrating macrophage density predicts poor prognosis in patients with primary hepatocellular carcinoma after resection. *Hum Pathol* 2009; **40**: 381-389 [PMID: 18992916 DOI: 10.1016/j.humpath.2008.08.011]

26   **Yeung OW**, Lo CM, Ling CC, Qi X, Geng W, Li CX, Ng KT, Forbes SJ, Guan XY, Poon RT, Fan ST, Man K. Alternatively activated (M2) macrophages promote tumour growth and invasiveness in hepatocellular carcinoma. *J Hepatol* 2015; **62**: 607-616 [PMID: 25450711 DOI: 10.1016/j.jhep.2014.10.029]

27   **Umemoto Y**, Okano S, Matsumoto Y, Nakagawara H, Matono R, Yoshiya S, Yamashita Y, Yoshizumi T, Ikegami T, Soejima Y, Harada M, Aishima S, Oda Y, Shirabe K, Maehara Y. Prognostic impact of programmed cell death 1 ligand 1 expression in human leukocyte antigen class I-positive hepatocellular carcinoma after curative hepatectomy. *J Gastroenterol* 2015; **50**: 65-75 [PMID: 24509608 DOI: 10.1007/s00535-014-0933-3]

28   **Kuang DM**, Zhao Q, Peng C, Xu J, Zhang JP, Wu C, Zheng L. Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. *J Exp Med* 2009; **206**: 1327-1337 [PMID: 19451266 DOI: 10.1084/jem.20082173]

29   **Zhou J**, Ding T, Pan W, Zhu LY, Li L, Zheng L. Increased intratumoral regulatory T cells are related to intratumoral macrophages and poor prognosis in hepatocellular carcinoma patients. *Int J Cancer* 2009; **125**: 1640-1648 [PMID: 19569243 DOI: 10.1002/ijc.24556]

30   **Wu Y**, Kuang DM, Pan WD, Wan YL, Lao XM, Wang D, Li XF, Zheng L. Monocyte/macrophage-elicited natural killer cell dysfunction in hepatocellular carcinoma is mediated by CD48/2B4 interactions. *Hepatology* 2013; **57**: 1107-1116 [PMID: 23225218 DOI: 10.1002/hep.26192]

31   **Yang J**, Zhang JX, Wang H, Wang GL, Hu QG, Zheng QC. Hepatocellular carcinoma and macrophage interaction induced tumor immunosuppression via Treg requires TLR4 signaling. *World J Gastroenterol* 2012; **18**: 2938-2947 [PMID: 22736917 DOI: 10.3748/wjg.v18.i23.2938]

32   **Capece D**, Fischietti M, Verzella D, Gaggiano A, Cicciarelli G, Tessitore A, Zazzeroni F, Alesse E. The inflammatory microenvironment in hepatocellular carcinoma: a pivotal role for tumor-associated macrophages. *Biomed Res Int* 2013; **2013**: 187204 [PMID: 23533994 DOI: 10.1155/2013/187204]

33   **Wan S**, Zhao E, Kryczek I, Vatan L, Sadovskaya A, Ludema G, Simeone DM, Zou W, Welling TH. Tumor-associated macrophages produce interleukin 6 and signal via STAT3 to promote expansion of human hepatocellular carcinoma stem cells. *Gastroenterology* 2014; **147**: 1393-1404 [PMID: 25181692 DOI: 10.1053/j.gastro.2014.08.039]

34   **Wan S**, Kuo N, Kryczek I, Zou W, Welling TH. Myeloid cells in hepatocellular carcinoma. *Hepatology* 2015; **62**: 1304-1312 [PMID: 25914264 DOI: 10.1002/hep.27867]

35   **Krenkel O**, Tacke F. Liver macrophages in tissue homeostasis and disease. *Nat Rev Immunol* 2017; **17**: 306-321 [PMID: 28317925 DOI: 10.1038/nri.2017.11]

36   **Wilson CL**, Jurk D, Fullard N, Banks P, Page A, Luli S, Elsharkawy AM, Gieling RG, Chakraborty JB, Fox C, Richardson C, Callaghan K, Blair GE, Fox N, Lagnado A, Passos JF, Moore AJ, Smith GR, Tiniakos DG, Mann J, Oakley F, Mann DA. NFκB1 is a suppressor of neutrophil-driven hepatocellular carcinoma. *Nat Commun* 2015; **6**: 6818 [PMID: 25879839 DOI: 10.1038/ncomms7818]

37   **Fridlender ZG**, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Worthen GS, Albelda SM. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell* 2009; **16**: 183-194 [PMID: 19732719 DOI: 10.1016/j.ccr.2009.06.017]

38   **Tsuda Y**, Fukui H, Asai A, Fukunishi S, Miyaji K, Fujiwara S, Teramura K, Fukuda A, Higuchi K. An immunosuppressive subtype of neutrophils identified in patients with hepatocellular carcinoma. *J Clin Biochem Nutr* 2012; **51**: 204-212 [PMID: 23170048 DOI: 10.3164/jcbn.12-32]

39   **He G**, Zhang H, Zhou J, Wang B, Chen Y, Kong Y, Xie X, Wang X, Fei R, Wei L, Chen H, Zeng H. Peritumoural neutrophils negatively regulate adaptive immunity via the PD-L1/PD-1 signalling

pathway in hepatocellular carcinoma. *J Exp Clin Cancer Res* 2015; **34**: 141 [PMID: 26581194 DOI: 10.1186/s13046-015-0256-0]

40   **Xiao WK**, Chen D, Li SQ, Fu SJ, Peng BG, Liang LJ. Prognostic significance of neutrophil-lymphocyte ratio in hepatocellular carcinoma: a meta-analysis. *BMC Cancer* 2014; **14**: 117 [PMID: 24559042 DOI: 10.1186/1471-2407-14-117]

41   **da Fonseca LG**, Barroso-Sousa R, Bento Ada S, Blanco BP, Valente GL, Pfiffer TE, Hoff PM, Sabbaga J. Pre-treatment neutrophil-to-lymphocyte ratio affects survival in patients with advanced hepatocellular carcinoma treated with sorafenib. *Med Oncol* 2014; **31**: 264 [PMID: 25273866 DOI: 10.1007/s12032-014-0264-5]

42   **Motomura T**, Shirabe K, Mano Y, Muto J, Toshima T, Umemoto Y, Fukuhara T, Uchiyama H, Ikegami T, Yoshizumi T, Soejima Y, Maehara Y. Neutrophil-lymphocyte ratio reflects hepatocellular carcinoma recurrence after liver transplantation via inflammatory microenvironment. *J Hepatol* 2013; **58**: 58-64 [PMID: 22925812 DOI: 10.1016/j.jhep.2012.08.017]

43   **Imai Y**, Kubota Y, Yamamoto S, Tsuji K, Shimatani M, Shibatani N, Takamido S, Matsushita M, Okazaki K. Neutrophils enhance invasion activity of human cholangiocellular carcinoma and hepatocellular carcinoma cells: an in vitro study. *J Gastroenterol Hepatol* 2005; **20**: 287-293 [PMID: 15683434 DOI: 10.1111/j.1440-1746.2004.03575.x]

44   **He M**, Peng A, Huang XZ, Shi DC, Wang JC, Zhao Q, Lin H, Kuang DM, Ke PF, Lao XM. Peritumoral stromal neutrophils are essential for c-Met-elicited metastasis in human hepatocellular carcinoma. *Oncoimmunology* 2016; **5**: e1219828 [PMID: 27853643 DOI: 10.1080/2162402x.2016.1219828]

45   **Li XF**, Chen DP, Ouyang FZ, Chen MM, Wu Y, Kuang DM, Zheng L. Increased autophagy sustains the survival and pro-tumourigenic effects of neutrophils in human hepatocellular carcinoma. *J Hepatol* 2015; **62**: 131-139 [PMID: 25152203 DOI: 10.1016/j.jhep.2014.08.023]

46   **Kuang DM**, Zhao Q, Wu Y, Peng C, Wang J, Xu Z, Yin XY, Zheng L. Peritumoral neutrophils link inflammatory response to disease progression by fostering angiogenesis in hepatocellular carcinoma. *J Hepatol* 2011; **54**: 948-955 [PMID: 21145847 DOI: 10.1016/j.jhep.2010.08.041]

47   **Xu R**, Huang H, Zhang Z, Wang FS. The role of neutrophils in the development of liver diseases. *Cell Mol Immunol* 2014; **11**: 224-231 [PMID: 24633014 DOI: 10.1038/cmi.2014.2]

48   **Guo CL**, Yang HC, Yang XH, Cheng W, Dong TX, Zhu WJ, Xu Z, Zhao L. Associations between infiltrating lymphocyte subsets and hepatocellular carcinoma. *Asian Pac J Cancer Prev* 2012; **13**: 5909-5913 [PMID: 23317279]

49   **Fu J**, Xu D, Liu Z, Shi M, Zhao P, Fu B, Zhang Z, Yang H, Zhang H, Zhou C, Yao J, Jin L, Wang H, Yang Y, Fu YX, Wang FS. Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. *Gastroenterology* 2007; **132**: 2328-2339 [PMID: 17570208 DOI: 10.1053/j.gastro.2007.03.102]

50   **Kalathil S**, Lugade AA, Miller A, Iyer R, Thanavala Y. Higher frequencies of GARP(+)CTLA-4(+)Foxp3(+) T regulatory cells and myeloid-derived suppressor cells in hepatocellular carcinoma patients are associated with impaired T-cell functionality. *Cancer Res* 2013; **73**: 2435-2444 [PMID: 23423978 DOI: 10.1158/0008-5472.can-12-3381]

51   **Garnelo M**, Tan A, Her Z, Yeong J, Lim CJ, Chen J, Lim KH, Weber A, Chow P, Chung A, Ooi LL, Toh HC, Heikenwalder M, Ng IO, Nardin A, Chen Q, Abastado JP, Chew V. Interaction between tumour-infiltrating B cells and T cells controls the progression of hepatocellular carcinoma. *Gut* 2017; **66**: 342-351 [PMID: 26669617 DOI: 10.1136/gutjnl-2015-310814]

52   **Ringelhan M**, Pfister D, O'Connor T, Pikarsky E, Heikenwalder M. The immunology of hepatocellular carcinoma. *Nature Immunology* 2018; **19**: 222-232 [DOI: 10.1038/s41590-018-0044-z]

53   **Yang S**, Tian Z, Wu Y, van Velkinburgh JC, Ni B. Pivotal roles of ILCs in hepatic diseases. *Int Rev Immunol* 2015; **34**: 509-522 [PMID: 25730441 DOI: 10.3109/08830185.2015.1008631]

54   **Artis D**, Spits H. The biology of innate lymphoid cells. *Nature* 2015; **517**: 293-301 [PMID: 25592534 DOI: 10.1038/nature14189]

55   **Abe H**, Kimura A, Tsuruta S, Fukaya T, Sakaguchi R, Morita R, Sekiya T, Shichita T, Chayama K, Fujii-Kuriyama Y, Yoshimura A. Aryl hydrocarbon receptor plays protective roles in ConA-induced hepatic injury by both suppressing IFN-γ expression and inducing IL-22. *Int Immunol* 2014; **26**: 129-137 [PMID: 24150244 DOI: 10.1093/intimm/dxt049]

56   **Kirchberger S**, Royston DJ, Boulard O, Thornton E, Franchini F, Szabady RL, Harrison O, Powrie F. Innate lymphoid cells sustain colon cancer through production of interleukin-22 in a mouse model. *J Exp Med* 2013; **210**: 917-931 [PMID: 23589566 DOI: 10.1084/jem.20122308]

57   **Zhang P**, Liu XK, Chu Z, Ye JC, Li KL, Zhuang WL, Yang DJ, Jiang YF. Detection of interleukin-33 in serum and carcinoma tissue from patients with hepatocellular carcinoma and its clinical implications. *J Int Med Res* 2012; **40**: 1654-1661 [PMID: 23206447 DOI: 10.1177/030006051204000504]

58   **Li J**, Razumilava N, Gores GJ, Walters S, Mizuochi T, Mourya R, Bessho K, Wang YH, Glaser SS, Shivakumar P, Bezerra JA. Biliary repair and carcinogenesis are mediated by IL-33-dependent cholangiocyte proliferation. *J Clin Invest* 2014; **124**: 3241-3251 [PMID: 24892809 DOI: 10.1172/jci73742]

59   **Jovanovic IP**, Pejnovic NN, Radosavljevic GD, Pantic JM, Milovanovic MZ, Arsenijevic NN, Lukic ML. Interleukin-33/ST2 axis promotes breast cancer growth and metastases by facilitating intratumoral accumulation of immunosuppressive and innate lymphoid cells. *Int J Cancer* 2014; **134**: 1669-1682 [PMID: 24105680 DOI: 10.1002/ijc.28481]

60   **McHedlidze T**, Waldner M, Zopf S, Walker J, Rankin AL, Schuchmann M, Voehringer D, McKenzie AN, Neurath MF, Pflanz S, Wirtz S. Interleukin-33-dependent innate lymphoid cells mediate hepatic fibrosis. *Immunity* 2013; **39**: 357-371 [PMID: 23954132 DOI: 10.1016/j.immuni.2013.07.018]

61   **Hanahan D**, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]

62   **Kettner NM**, Voicu H, Finegold MJ, Coarfa C, Sreekumar A, Putluri N, Katchy CA, Lee C, Moore DD, Fu L. Circadian Homeostasis of Liver Metabolism Suppresses Hepatocarcinogenesis. *Cancer Cell* 2016; **30**: 909-924 [PMID: 27889186 DOI: 10.1016/j.ccell.2016.10.007]

63   **Huang Q**, Tan Y, Yin P, Ye G, Gao P, Lu X, Wang H, Xu G. Metabolic characterization of hepatocellular carcinoma using nontargeted tissue metabolomics. *Cancer Res* 2013; **73**: 4992-5002 [PMID: 23824744 DOI: 10.1158/0008-5472.CAN-13-0308]

64   **Gingold JA**, Zhu D, Lee DF, Kaseb A, Chen J. Genomic Profiling and Metabolic Homeostasis in Primary Liver Cancers. *Trends Mol Med* 2018; **24**: 395-411 [PMID: 29530485 DOI: 10.1016/j.molmed.2018.02.006]

65   **Ngo H**, Tortorella SM, Ververis K, Karagiannis TC. The Warburg effect: molecular aspects and therapeutic possibilities. *Mol Biol Rep* 2015; **42**: 825-834 [PMID: 25253100 DOI: 10.1007/s11033-014-3764-7]

66   **Jones RG**, Thompson CB. Tumor suppressors and cell metabolism: a recipe for cancer growth. *Genes Dev* 2009; **23**: 537-548 [PMID: 19270154 DOI: 10.1101/gad.1756509]

67   **Shang RZ**, Qu SB, Wang DS. Reprogramming of glucose metabolism in hepatocellular carcinoma: Progress and prospects. *World J Gastroenterol* 2016; **22**: 9933-9943 [PMID: 28018100 DOI: 10.3748/wjg.v22.i45.9933]

68   **Zhang Q**, Bai X, Chen W, Ma T, Hu Q, Liang C, Xie S, Chen C, Hu L, Xu S, Liang T. Wnt/β-catenin signaling enhances hypoxia-induced epithelial-mesenchymal transition in hepatocellular carcinoma via crosstalk with hif-1α signaling. *Carcinogenesis* 2013; **34**: 962-973 [PMID: 23358852 DOI: 10.1093/carcin/bgt027]

69   **Kitamura K**, Hatano E, Higashi T, Narita M, Seo S, Nakamoto Y, Yamanaka K, Nagata H, Taura K, Yasuchika K, Nitta T, Uemoto S. Proliferative activity in hepatocellular carcinoma is closely correlated with glucose metabolism but not angiogenesis.

J Hepatol 2011; **55**: 846-857 [PMID: 21334407 DOI: 10.1016/j.jhep.2011.01.038]

70   **Jia YY**, Zhao JY, Li BL, Gao K, Song Y, Liu MY, Yang XJ, Xue Y, Wen AD, Shi L. miR-592/WSB1/HIF-1α axis inhibits glycolytic metabolism to decrease hepatocellular carcinoma growth. *Oncotarget* 2016; **7**: 35257-35269 [PMID: 27153552 DOI: 10.18632/oncotarget.9135]

71   **Jiang J**, Nilsson-Ehle P, Xu N. Influence of liver cancer on lipid and lipoprotein metabolism. *Lipids Health Dis* 2006; **5**: 4 [PMID: 16515689 DOI: 10.1186/1476-511x-5-4]

72   **Budhu A**, Roessler S, Zhao X, Yu Z, Forgues M, Ji J, Karoly E, Qin LX, Ye QH, Jia HL, Fan J, Sun HC, Tang ZY, Wang XW. Integrated metabolite and gene expression profiles identify lipid biomarkers associated with progression of hepatocellular carcinoma and patient outcomes. *Gastroenterology* 2013; **144**: 1066-1075.e1 [PMID: 23376425 DOI: 10.1053/j.gastro.2013.01.054]

73   **Yang H**, Ye D, Guan KL, Xiong Y. IDH1 and IDH2 mutations in tumorigenesis: mechanistic insights and clinical perspectives. *Clin Cancer Res* 2012; **18**: 5562-5571 [PMID: 23071358 DOI: 10.1158/1078-0432.CCR-12-1773]

74   **Turkalp Z**, Karamchandani J, Das S. IDH mutation in glioma: new insights and promises for the future. *JAMA Neurol* 2014; **71**: 1319-1325 [PMID: 25155243 DOI: 10.1001/jamaneurol.2014.1205]

75   **Borger DR**, Goyal L, Yau T, Poon RT, Ancukiewicz M, Deshpande V, Christiani DC, Liebman HM, Yang H, Kim H, Yen K, Faris JE, Iafrate AJ, Kwak EL, Clark JW, Allen JN, Blaszkowsky LS, Murphy JE, Saha SK, Hong TS, Wo JY, Ferrone CR, Tanabe KK, Bardeesy N, Straley KS, Agresta S, Schenkein DP, Ellisen LW, Ryan DP, Zhu AX. Circulating oncometabolite 2-hydroxyglutarate is a potential surrogate biomarker in patients with isocitrate dehydrogenase-mutant intrahepatic cholangiocarcinoma. *Clin Cancer Res* 2014; **20**: 1884-1890 [PMID: 24478380 DOI: 10.1158/1078-0432.CCR-13-2649]

76   **Fathi AT**, Nahed BV, Wander SA, Iafrate AJ, Borger DR, Hu R, Thabet A, Cahill DP, Perry AM, Joseph CP, Muzikansky A, Chi AS. Elevation of Urinary 2-Hydroxyglutarate in IDH-Mutant Glioma. *Oncologist* 2016; **21**: 214-219 [PMID: 26834160 DOI: 10.1634/theoncologist.2015-0342]

77   **Chen JY**, Lai YS, Tsai HJ, Kuo CC, Yen BL, Yeh SP, Sun HS, Hung WC. The oncometabolite R-2-hydroxyglutarate activates NF-κB-dependent tumor-promoting stromal niche for acute myeloid leukemia cells. *Sci Rep* 2016; **6**: 32428 [PMID: 27577048 DOI: 10.1038/srep32428]

78   **Zhang C**, Moore LM, Li X, Yung WK, Zhang W. IDH1/2 mutations target a key hallmark of cancer by deregulating cellular metabolism in glioma. *Neuro Oncol* 2013; **15**: 1114-1126 [PMID: 23877318 DOI: 10.1093/neuonc/not087]

79   **Izquierdo-Garcia JL**, Viswanath P, Eriksson P, Cai L, Radoul M, Chaumeil MM, Blough M, Luchman HA, Weiss S, Cairncross JG, Phillips JJ, Pieper RO, Ronen SM. IDH1 Mutation Induces Reprogramming of Pyruvate Metabolism. *Cancer Res* 2015; **75**: 2999-3009 [PMID: 26045167 DOI: 10.1158/0008-5472.can-15-0840]

80   **Bogdanovic E**. IDH1, lipid metabolism and cancer: Shedding new light on old ideas. *Biochim Biophys Acta* 2015; **1850**: 1781-1785 [PMID: 25960387 DOI: 10.1016/j.bbagen.2015.04.014]

81   **Arai Y**, Totoki Y, Hosoda F, Shirota T, Hama N, Nakamura H, Ojima H, Furuta K, Shimada K, Okusaka T, Kosuge T, Shibata T. Fibroblast growth factor receptor 2 tyrosine kinase fusions define a unique molecular subtype of cholangiocarcinoma. *Hepatology* 2014; **59**: 1427-1434 [PMID: 24122810 DOI: 10.1002/hep.26890]

82   **Cheng AL**, Shen YC, Zhu AX. Targeting fibroblast growth factor receptor signaling in hepatocellular carcinoma. *Oncology* 2011; **81**: 372-380 [PMID: 22269894 DOI: 10.1159/000335472]

83   **Churi CR**, Shroff R, Wang Y, Rashid A, Kang HC, Weatherly J, Zuo M, Zinner R, Hong D, Meric-Bernstam F, Janku F, Crane CH, Mishra L, Vauthey JN, Wolff RA, Mills G, Javle M. Mutation profiling in cholangiocarcinoma: prognostic and therapeutic implications. *PLoS One* 2014; **9**: e115383 [PMID: 25536104 DOI: 10.1371/journal.pone.0115383]

84   **Sia D**, Tovar V, Moeini A, Llovet JM. Intrahepatic cholangiocarcinoma: pathogenesis and rationale for molecular therapies. *Oncogene* 2013; **32**: 4861-4870 [PMID: 23318457 DOI: 10.1038/onc.2012.617]

85   **Wu YM**, Su F, Kalyana-Sundaram S, Khazanov N, Ateeq B, Cao X, Lonigro RJ, Vats P, Wang R, Lin SF, Cheng AJ, Kunju LP, Siddiqui J, Tomlins SA, Wyngaard P, Sadis S, Roychowdhury S, Hussain MH, Feng FY, Zalupski MM, Talpaz M, Pienta KJ, Rhodes DR, Robinson DR, Chinnaiyan AM. Identification of targetable FGFR gene fusions in diverse cancers. *Cancer Discov* 2013; **3**: 636-647 [PMID: 23558953 DOI: 10.1158/2159-8290.cd-13-0050]

86   **Frattini V**, Pagnotta SM, Tala, Fan JJ, Russo MV, Lee SB, Garofano L, Zhang J, Shi P, Lewis G, Sanson H, Frederick V, Castano AM, Cerulo L, Rolland DCM, Mall R, Mokhtari K, Elenitoba-Johnson KSJ, Sanson M, Huang X, Ceccarelli M, Lasorella A, Iavarone A. A metabolic function of FGFR3-TACC3 gene fusions in cancer. *Nature* 2018; **553**: 222-227 [PMID: 29323298 DOI: 10.1038/nature25171]

87   **Chiang CH**, Huang KC. Association between metabolic factors and chronic hepatitis B virus infection. *World J Gastroenterol* 2014; **20**: 7213-7216 [PMID: 24966591 DOI: 10.3748/wjg.v20.i23.7213]

88   **Schoeman JC**, Hou J, Harms AC, Vreeken RJ, Berger R, Hankemeier T, Boonstra A. Metabolic characterization of the natural progression of chronic hepatitis B. *Genome Med* 2016; **8**: 64 [PMID: 27286979 DOI: 10.1186/s13073-016-0318-8]

89   **Agani F**, Jiang BH. Oxygen-independent regulation of HIF-1: novel involvement of PI3K/AKT/mTOR pathway in cancer. *Curr Cancer Drug Targets* 2013; **13**: 245-251 [PMID: 23297826]

90   **Kasuno K**, Takabuchi S, Fukuda K, Kizaka-Kondoh S, Yodoi J, Adachi T, Semenza GL, Hirota K. Nitric oxide induces hypoxia-inducible factor 1 activation that is dependent on MAPK and phosphatidylinositol 3-kinase signaling. *J Biol Chem* 2004; **279**: 2550-2558 [PMID: 14600153 DOI: 10.1074/jbc.M308197200]

91   **Tanaka H**, Yamamoto M, Hashimoto N, Miyakoshi M, Tamakawa S, Yoshie M, Tokusashi Y, Yokoyama K, Yaginuma Y, Ogawa K. Hypoxia-independent overexpression of hypoxia-inducible factor 1alpha as an early change in mouse hepatocarcinogenesis. *Cancer Res* 2006; **66**: 11263-11270 [PMID: 17145871 DOI: 10.1158/0008-5472.can-06-1699]

92   **Xia L**, Mo P, Huang W, Zhang L, Wang Y, Zhu H, Tian D, Liu J, Chen Z, Zhang Y, Chen Z, Hu H, Fan D, Nie Y, Wu K. The TNF-α/ROS/HIF-1-induced upregulation of FoxMI expression promotes HCC proliferation and resistance to apoptosis. *Carcinogenesis* 2012; **33**: 2250-2259 [PMID: 22831955 DOI: 10.1093/carcin/bgs249]

93   **Wang MD**, Wu H, Huang S, Zhang HL, Qin CJ, Zhao LH, Fu GB, Zhou X, Wang XM, Tang L, Wen W, Yang W, Tang SH, Cao D, Guo LN, Zeng M, Wu MC, Yan HX, Wang HY. HBx regulates fatty acid oxidation to promote hepatocellular carcinoma survival during metabolic stress. *Oncotarget* 2016; **7**: 6711-6726 [PMID: 26744319 DOI: 10.18632/oncotarget.6817]

94   **Kim K**, Kim KH, Kim HH, Cheong J. Hepatitis B virus X protein induces lipogenic transcription factor SREBP1 and fatty acid synthase through the activation of nuclear receptor LXRalpha. *Biochem J* 2008; **416**: 219-230 [PMID: 18782084 DOI: 10.1042/bj20081336]

95   **Kim SY**, Kim JK, Kim HJ, Ahn JK. Hepatitis B virus X protein sensitizes UV-induced apoptosis by transcriptional transactivation of Fas ligand gene expression. *IUBMB Life* 2005; **57**: 651-658 [PMID: 16203685 DOI: 10.1080/15216540500239697]

96   **Dai CY**, Yeh ML, Huang CF, Hou CH, Hsieh MY, Huang JF, Lin IL, Lin ZY, Chen SC, Wang LY, Chuang WL, Yu ML, Tung HD. Chronic hepatitis C infection is associated with insulin resistance and lipid profiles. *J Gastroenterol Hepatol* 2015; **30**: 879-884 [PMID: 23808794 DOI: 10.1111/jgh.12313]

97   **Kawaguchi Y**, Mizuta T. Interaction between hepatitis C virus and metabolic factors. *World J Gastroenterol* 2014; **20**: 2888-2901 [PMID: 24659880 DOI: 10.3748/wjg.v20.i11.2888]

98   **Bernsmeier C**, Calabrese D, Heim MH, Duong HT. Hepatitis C virus dysregulates glucose homeostasis by a dual mechanism involving induction of PGC1α and dephosphorylation of FoxO1. *J Viral Hepat* 2014; **21**: 9-18 [PMID: 24329853 DOI: 10.1111/jvh.12208]

99   **Lee S**, Mardinoglu A, Zhang C, Lee D, Nielsen J. Dysregulated signaling hubs of liver lipid metabolism reveal hepatocellular carcinoma pathogenesis. *Nucleic Acids Res* 2016; **44**: 5529-5539 [PMID: 27216817 DOI: 10.1093/nar/gkw462]

100   **Sanyal AJ**, Campbell-Sargent C, Mirshahi F, Rizzo WB, Contos MJ, Sterling RK, Luketic VA, Shiffman ML, Clore JN. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* 2001; **120**: 1183-1192 [PMID: 11266382 DOI: 10.1053/gast.2001.23256]

101   **Satapati S**, Kucejova B, Duarte JA, Fletcher JA, Reynolds L, Sunny NE, He T, Nair LA, Livingston KA, Fu X, Merritt ME, Sherry AD, Malloy CR, Shelton JM, Lambert J, Parks EJ, Corbin I, Magnuson MA, Browning JD, Burgess SC. Mitochondrial metabolism mediates oxidative stress and inflammation in fatty liver. *J Clin Invest* 2015; **125**: 4447-4462 [PMID: 26571396 DOI: 10.1172/JCI82204]

102   **Nassir F**, Ibdah JA. Role of mitochondria in nonalcoholic fatty liver disease. *Int J Mol Sci* 2014; **15**: 8713-8742 [PMID: 24837835 DOI: 10.3390/ijms15058713]

103   **Fromenty B**, Robin MA, Igoudjil A, Mansouri A, Pessayre D. The ins and outs of mitochondrial dysfunction in NASH. *Diabetes Metab* 2004; **30**: 121-138 [PMID: 15223984]

104   **Fujisawa K**, Takami T, Matsumoto T, Yamamoto N, Sakaida I. Profiling of the circadian metabolome in thioacetamide-induced liver cirrhosis in mice. *Hepatol Commun* 2017; **1**: 704-718 [PMID: 29404487 DOI: 10.1002/hep4.1075]

105   **Nishikawa T**, Bellance N, Damm A, Bing H, Zhu Z, Handa K, Yovchev MI, Sehgal V, Moss TJ, Oertel M, Ram PT, Pipinos II, Soto-Gutierrez A, Fox IJ, Nagrath D. A switch in the source of ATP production and a loss in capacity to perform glycolysis are hallmarks of hepatocyte failure in advance liver disease. *J Hepatol* 2014; **60**: 1203-1211 [PMID: 24583248 DOI: 10.1016/j.jhep.2014.02.014]

106   **Andrejeva G**, Rathmell JC. Similarities and Distinctions of Cancer and Immune Metabolism in Inflammation and Tumors. *Cell Metab* 2017; **26**: 49-70 [PMID: 28683294 DOI: 10.1016/j.cmet.2017.06.004]

107   **Haschemi A**, Kosma P, Gille L, Evans CR, Burant CF, Starkl P, Knapp B, Haas R, Schmid JA, Jandl C, Amir S, Lubec G, Park J, Esterbauer H, Bilban M, Brizuela L, Pospisilik JA, Otterbein LE, Wagner O. The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism. *Cell Metab* 2012; **15**: 813-826 [PMID: 22682222 DOI: 10.1016/j.cmet.2012.04.023]

108   **Huang SC**, Everts B, Ivanova Y, O'Sullivan D, Nascimento M, Smith AM, Beatty W, Love-Gregory L, Lam WY, O'Neill CM, Yan C, Du H, Abumrad NA, Urban JF Jr, Artyomov MN, Pearce EL, Pearce EJ. Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. *Nat Immunol* 2014; **15**: 846-855 [PMID: 25086775 DOI: 10.1038/ni.2956]

109   **Namgaladze D**, Brüne B. Fatty acid oxidation is dispensable for human macrophage IL-4-induced polarization. *Biochim Biophys Acta* 2014; **1841**: 1329-1335 [PMID: 24960101 DOI: 10.1016/j.bbalip.2014.06.007]

110   **Nomura M**, Liu J, Rovira II, Gonzalez-Hurtado E, Lee J, Wolfgang MJ, Finkel T. Fatty acid oxidation in macrophage polarization. *Nat Immunol* 2016; **17**: 216-217 [PMID: 26882249 DOI: 10.1038/ni.3366]

111   **Zhang Q**, Wang HR, Mao CY, Sun M, Dominah G, Chen L, Zhuang Z. Fatty acid oxidation contributes to IL-1β secretion in M2 macrophages and promotes macrophage-mediated tumor cell migration. *Mol Metab* 2018; **94**: 27-35 [DOI: 10.1016/j.molimm.2017.12.011]

112   **Vats D**, Mukundan L, Odegaard JI, Zhang L, Smith KL, Morel CR, Wagner RA, Greaves DR, Murray PJ, Chawla A. Oxidative metabolism and PGC-1beta attenuate macrophage-mediated inflammation. *Cell Metab* 2006; **4**: 13-24 [PMID: 16814729 DOI: 10.1016/j.cmet.2006.05.011]

113   **Biswas SK**. Metabolic Reprogramming of Immune Cells in Cancer Progression. *Immunity* 2015; **43**: 435-449 [PMID: 26377897 DOI: 10.1016/j.immuni.2015.09.001]

114   **Covarrubias AJ**, Aksoylar HI, Yu J, Snyder NW, Worth AJ, Iyer SS, Wang J, Ben-Sahra I, Byles V, Polynne-Stapornkul T, Espinosa EC, Lamming D, Manning BD, Zhang Y, Blair IA, Horng T. Akt-mTORC1 signaling regulates Acly to integrate metabolic input to control of macrophage activation. *Elife* 2016; **5** [PMID: 26894960 DOI: 10.7554/eLife.11612]

115   **Lim JE**, Chung E, Son Y. A neuropeptide, Substance-P, directly induces tissue-repairing M2 like macrophages by activating the PI3K/Akt/mTOR pathway even in the presence of IFNγ. *Sci Rep* 2017; **7**: 9417 [PMID: 28842601 DOI: 10.1038/s41598-017-09639-7]

116   **Zhou L**, Wang Q, Yin P, Xing W, Wu Z, Chen S, Lu X, Zhang Y, Lin X, Xu G. Serum metabolomics reveals the deregulation of fatty acids metabolism in hepatocellular carcinoma and chronic liver diseases. *Anal Bioanal Chem* 2012; **403**: 203-213 [PMID: 22349331 DOI: 10.1007/s00216-012-5782-4]

117   **Nath A**, Li I, Roberts LR, Chan C. Elevated free fatty acid uptake via CD36 promotes epithelial-mesenchymal transition in hepatocellular carcinoma. *Sci Rep* 2015; **5**: 14752 [PMID: 26424075 DOI: 10.1038/srep14752]

118   **Feingold KR**, Shigenaga JK, Kazemi MR, McDonald CM, Patzek SM, Cross AS, Moser A, Grunfeld C. Mechanisms of triglyceride accumulation in activated macrophages. *J Leukoc Biol* 2012; **92**: 829-839 [PMID: 22753953 DOI: 10.1189/jlb.1111537]

119   **Colegio OR**, Chu NQ, Szabo AL, Chu T, Rhebergen AM, Jairam V, Cyrus N, Brokowski CE, Eisenbarth SC, Phillips GM, Cline GW, Phillips AJ, Medzhitov R. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature* 2014; **513**: 559-563 [PMID: 25043024 DOI: 10.1038/nature13490]

120   **Ruan GX**, Kazlauskas A. Lactate engages receptor tyrosine kinases Axl, Tie2, and vascular endothelial growth factor receptor 2 to activate phosphoinositide 3-kinase/Akt and promote angiogenesis. *J Biol Chem* 2013; **288**: 21161-21172 [PMID: 23754286 DOI: 10.1074/jbc.M113.474619]

121   **Ohashi T**, Aoki M, Tomita H, Akazawa T, Sato K, Kuze B, Mizuta K, Hara A, Nagaoka H, Inoue N, Ito Y. M2-like macrophage polarization in high lactic acid-producing head and neck cancer. *Cancer Sci* 2017; **108**: 1128-1134 [PMID: 28370718 DOI: 10.1111/cas.13244]

122   **Spadaro O**, Camell CD, Bosurgi L, Nguyen KY, Youm YH, Rothlin CV, Dixit VD. IGF1 Shapes Macrophage Activation in Response to Immunometabolic Challenge. *Cell Rep* 2017; **19**: 225-234 [PMID: 28402847 DOI: 10.1016/j.celrep.2017.03.046]

123   **Pearce EL**, Pearce EJ. Metabolic pathways in immune cell activation and quiescence. *Immunity* 2013; **38**: 633-643 [PMID: 23601682 DOI: 10.1016/j.immuni.2013.04.005]

124   **Palsson-McDermott EM**, Curtis AM, Goel G, Lauterbach MA, Sheedy FJ, Gleeson LE, van den Bosch MW, Quinn SR, Domingo-Fernandez R, Johnston DG, Jiang JK, Israelsen WJ, Keane J, Thomas C, Clish C, Vander Heiden M, Xavier RJ, O'Neill LA. Pyruvate kinase M2 regulates Hif-1α activity and IL-1β induction and is a critical determinant of the warburg effect in LPS-activated macrophages. *Cell Metab* 2015; **21**: 65-80 [PMID: 25565206 DOI: 10.1016/j.cmet.2014.12.005]

125   **Moon JS**, Nakahira K, Chung KP, DeNicola GM, Koo MJ, Pabón MA, Rooney KT, Yoon JH, Ryter SW, Stout-Delgado H, Choi AM. NOX4-dependent fatty acid oxidation promotes NLRP3 inflammasome activation in macrophages. *Nat Med* 2016; **22**: 1002-1012 [PMID: 27455510 DOI: 10.1038/nm.4153]

126   **Moon JS**, Hisata S, Park MA, DeNicola GM, Ryter SW, Nakahira K, Choi AMK. mTORC1-Induced HK1-Dependent Glycolysis Regulates NLRP3 Inflammasome Activation. *Cell Rep* 2015; **12**: 102-115 [PMID: 26119735 DOI: 10.1016/j.celrep.2015.05.046]

127   **Littlewood-Evans A**, Sarret S, Apfel V, Loesle P, Dawson J, Zhang

J, Muller A, Tigani B, Kneuer R, Patel S, Valeaux S, Gommermann N, Rubic-Schneider T, Junt T, Carballido JM. GPR91 senses extracellular succinate released from inflammatory macrophages and exacerbates rheumatoid arthritis. *J Exp Med* 2016; **213**: 1655-1662 [PMID: 27481132 DOI: 10.1084/jem.20160061]

128 **Mills EL**, Kelly B, Logan A, Costa ASH, Varma M, Bryant CE, Tourlomousis P, Däbritz JHM, Gottlieb E, Latorre I, Corr SC, McManus G, Ryan D, Jacobs HT, Szibor M, Xavier RJ, Braun T, Frezza C, Murphy MP, O'Neill LA. Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages. *Cell* 2016; **167**: 457-470.e13 [PMID: 27667687 DOI: 10.1016/j.cell.2016.08.064]

129 **Tannahill GM**, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, Frezza C, Bernard NJ, Kelly B, Foley NH, Zheng L, Gardet A, Tong Z, Jany SS, Corr SC, Haneklaus M, Caffrey BE, Pierce K, Walmsley S, Beasley FC, Cummins E, Nizet V, Whyte M, Taylor CT, Lin H, Masters SL, Gottlieb E, Kelly VP, Clish C, Auron PE, Xavier RJ, O'Neill LA. Succinate is an inflammatory signal that induces IL-1β through HIF-1α. *Nature* 2013; **496**: 238-242 [PMID: 23535595 DOI: 10.1038/nature11986]

130 **Azevedo EP**, Rochael NC, Guimarães-Costa AB, de Souza-Vieira TS, Ganilho J, Saraiva EM, Palhano FL, Foguel D. A Metabolic Shift toward Pentose Phosphate Pathway Is Necessary for Amyloid Fibril- and Phorbol 12-Myristate 13-Acetate-induced Neutrophil Extracellular Trap (NET) Formation. *J Biol Chem* 2015; **290**: 22174-22183 [PMID: 26198639 DOI: 10.1074/jbc.M115.640094]

131 **Siska PJ**, Rathmell JC. T cell metabolic fitness in antitumor immunity. *Trends Immunol* 2015; **36**: 257-264 [PMID: 25773310 DOI: 10.1016/j.it.2015.02.007]

132 **Chang CH**, Curtis JD, Maggi LB Jr, Faubert B, Villarino AV, O'Sullivan D, Huang SC, van der Windt GJ, Blagih J, Qiu J, Weber JD, Pearce EJ, Jones RG, Pearce EL. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* 2013; **153**: 1239-1251 [PMID: 23746840 DOI: 10.1016/j.cell.2013.05.016]

133 **Zhang Y**, Kurupati R, Liu L, Zhou XY, Zhang G, Hudaihed A, Filisio F, Giles-Davis W, Xu X, Karakousis GC, Schuchter LM, Xu W, Amaravadi R, Xiao M, Sadek N, Krepler C, Herlyn M, Freeman GJ, Rabinowitz JD, Ertl HCJ. Enhancing CD8+ T Cell Fatty Acid Catabolism within a Metabolically Challenging Tumor Microenvironment Increases the Efficacy of Melanoma Immunotherapy. *Cancer Cell* 2017; **32**: 377-391.e9 [PMID: 28898698 DOI: 10.1016/j.ccell.2017.08.004]

134 **Wawman RE**, Bartlett H, Oo YH. Regulatory T Cell Metabolism in the Hepatic Microenvironment. *Front Immunol* 2018; **8**: 1889 [PMID: 29358934 DOI: 10.3389/fimmu.2017.01889]

135 **Beier UH**, Angelin A, Akimova T, Wang L, Liu Y, Xiao H, Koike MA, Hancock SA, Bhatti TR, Han R, Jiao J, Veasey SC, Sims CA, Baur JA, Wallace DC, Hancock WW. Essential role of mitochondrial energy metabolism in Foxp3+ T-regulatory cell function and allograft survival. *FASEB J* 2015; **29**: 2315-2326 [PMID: 25681462 DOI: 10.1096/fj.14-268409]

136 **Shirabe K**, Mano Y, Muto J, Matono R, Motomura T, Toshima T, Takeishi K, Uchiyama H, Yoshizumi T, Taketomi A, Morita M, Tsujitani S, Sakaguchi Y, Maehara Y. Role of tumor-associated macrophages in the progression of hepatocellular carcinoma. *Surg Today* 2012; **42**: 1-7 [PMID: 22116397 DOI: 10.1007/s00595-011-0058-8]

137 **Zhang J**, Zhang Q, Lou Y, Fu Q, Chen Q, Wei T, Yang J, Tang J, Wang J, Chen Y, Zhang X, Zhang J, Bai X, Liang T. Hypoxia-inducible factor-1α/interleukin-1β signaling enhances hepatoma epithelial-mesenchymal transition through macrophages in a hypoxic-inflammatory microenvironment. *Hepatology* 2018; **67**: 1872-1889 [PMID: 29171040 DOI: 10.1002/hep.29681]

138 **Clambey ET**, McNamee EN, Westrich JA, Glover LE, Campbell EL, Jedlicka P, de Zoeten EF, Cambier JC, Stenmark KR, Colgan SP, Eltzschig HK. Hypoxia-inducible factor-1 alpha-dependent induction of FoxP3 drives regulatory T-cell abundance and function during inflammatory hypoxia of the mucosa. *Proc Natl Acad Sci* 2012; **109**: E2784-E2793 [PMID: 22988108 DOI: 10.1073/pnas.1202366109]

139 **Chang M**, Hamilton JA, Scholz GM, Elsegood CL. Glycolytic control of adjuvant-induced macrophage survival: role of PI3K, MEK1/2, and Bcl-2. *J Leukoc Biol* 2009; **85**: 947-956 [PMID: 19270084 DOI: 10.1189/jlb.0908522]

140 **Sharma MD**, Shinde R, McGaha TL, Huang L, Holmgaard RB, Wolchok JD, Mautino MR, Celis E, Sharpe AH, Francisco LM, Powell JD, Yagita H, Mellor AL, Blazar BR, Munn DH. The PTEN pathway in Tregs is a critical driver of the suppressive tumor microenvironment. *Sci Adv* 2015; **1**: e1500845 [PMID: 26601142 DOI: 10.1126/sciadv.1500845]

141 **Chen W**, Ma T, Shen XN, Xia XF, Xu GD, Bai XL, Liang TB. Macrophage-induced tumor angiogenesis is regulated by the TSC2-mTOR pathway. *Cancer Res* 2012; **72**: 1363-1372 [PMID: 22287548 DOI: 10.1158/0008-5472.can-11-2684]

142 **Zeng H**, Yang K, Cloer C, Neale G, Vogel P, Chi H. mTORC1 couples immune signals and metabolic programming to establish T(reg)-cell function. *Nature* 2013; **499**: 485-490 [PMID: 23812589 DOI: 10.1038/nature12297]

Osteoporosis in primary biliary cholangitis

Christopher J Danford, Hirsh D Trivedi, Konstantinos Papamichael, Elliot B Tapper, Alan Bonder

Christopher J Danford, Hirsh D Trivedi, Konstantinos Papamichael, Alan Bonder, Division of Gastroenterology and Hepatology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, Unites States

Elliot B Tapper, Department of Hepatology, University of Michigan, Ann Arbor, MI 48109, Unites States

ORCID number: Christopher J Danford (0000-0003-0322-7705); Hirsh D Trivedi (0000-0002-8328-9211); Konstantinos Papamichael (0000-0003-1497-0254); Elliot B Tapper (0000-0002-0839-1515); Alan Bonder (0000-0001-6821-0416)

Correspondence to: Alan Bonder, MD, Attending Doctor, Division of Gastroenterology and Hepatology, Beth Israel Deaconess Medical Center, 110 Francis St. Suite 8E, Boston, MA 02215, United States. abonder@bidmc.harvard.edu
Telephone: +1-617-6321070
Fax: +1-617-6231065

Abstract

Primary biliary cholangitis (PBC) is an autoimmune cholestatic liver disease with multiple debilitating complications. Osteoporosis is a common complication of PBC resulting in frequent fractures and leading to significant morbidity in this population, yet evidence for effective therapy is lacking. We sought to summarize our current understanding of the pathophysiology of osteoporosis in PBC, as well as current and emerging therapies in order to guide future research directions. A complete search with a comprehensive literature review was performed with studies from PubMed, EMBASE, Web of Science, Cochrane database, and the Countway Library. Osteoporosis in PBC is driven primarily by decreased bone formation, which differs from the increased bone resorption seen in postmenopausal osteoporosis. Despite this fundamental difference, current treatment recommendations are based primarily on experience with postmenopausal osteoporosis. Trials specific to PBC-related osteoporosis are small and have not consistently demonstrated a benefit in this population. As it stands, prevention of osteoporosis in PBC relies on the mitigation of risk factors such as smoking and alcohol use, as well as encouraging a healthy diet and weight-bearing exercise. The primary medical intervention for the treatment of osteoporosis in PBC remains bisphosphonates though a benefit in terms of fracture reduction has never been shown. This review outlines what is known regarding the pathogenesis of bone disease in PBC and summarizes current and emerging therapies.

Key words: Biliary cirrhosis; Cholestatic liver disease; Osteopenia; Hepatic osteodystrophy; Bisphosphonates; Hormone replacement therapy

Core tip: This article reviews the available literature on the pathophysiology and management of osteoporosis in primary biliary cholangitis (PBC). PBC-related osteoporosis is driven mainly by decreased bone formation as opposed to the increased bone resorption seen in postmenopausal osteoporosis. Despite this and a lack of evidence of efficacy, bisphosphonates remain the cornerstone of treatment. Future attention should be given to the use of anabolic bone agents in the treatment of PBC-related osteoporosis.

INTRODUCTION

Primary biliary cholangitis (PBC) is a chronic, progressive cholestatic liver disease associated with numerous extrahepatic complications. Osteoporosis, a disease of decreased bone density and strength, is a common complication of PBC[1-3]. The prevalence of osteoporosis in patients with PBC is roughly 30%[1-3] and higher in advanced stages of liver disease, up to 44% in those awaiting liver transplantation[4]. Osteoporosis is a major risk factor for fractures[3,5,6]. The incidence of fractures in PBC ranges from 0-14% over a two-year period[6-9]. The prevalence of fractures is reported to be 10%-20%[3,5,6], increasing to 22% in transplant waitlisted patients[4]. Serious injuries such as fractures are more difficult to manage in patients with advanced liver disease with perioperative morbidity and mortality approaching 80% and 60% respectively in cirrhotics undergoing emergent total hip arthroplasty[10]. Patients would therefore benefit from a timely diagnosis, effective risk factor modification, and treatment of PBC-related osteoporosis.

While effective treatments exist for postmenopausal osteoporosis, management of osteoporosis in PBC is limited by an incomplete understanding of pathophysiology specific to this disease process and a paucity of studies evaluating potential treatment. The current American Association for the Study of Liver Diseases guidelines recommend vitamin D and calcium supplementation based on experience in postmenopausal women as well as alendronate based on a single RCT in PBC[11]. An overreliance on postmenopausal osteoporosis data may lead to relatively ineffective treatment of PBC-related osteoporosis. To this end, we aim to summarize our current understanding of the pathophysiology behind bone disease in PBC as well as the evidence behind current and emerging therapies for osteoporosis in PBC.

The term hepatic osteodystrophy is sometimes used to refer to all metabolic bone disease seen in chronic liver disease. It refers to both osteomalacia, or decreased bone mineralization, and osteoporosis, or decreased bone mass, which can both be seen in advanced liver disease. Osteomalacia was once thought to be common in cholestatic liver disease; theoretically due dietary vitamin D malabsorption in the setting of severe cholestasis and impaired hepatic 25-hydroxylation[12,13]. However, it appears osteomalacia is actually quite rare in PBC and cholestatic liver disease and early studies were plagued by selection bias and a loose definition of osteomalacia[14]. It is now widely accepted that osteoporosis is the primary metabolic bone disease in PBC[15] and this review will focus on the management of osteoporosis in PBC.

Pathogenesis

The pathogenesis of osteoporosis in PBC appears to be largely driven by decreased bone formation, though increased bone resorption may play a role in certain scenarios. Several studies evaluating bone histomorphometry have shown decreased tetracycline double-labeling, decreased bone formation rates, decreased osteoblast numbers, and decreased serum osteocalcin, a marker of bone formation, all pointing towards osteoblast dysfunction and deficient bone formation as central to the pathogenesis of PBC-related osteoporosis[5,16-18].

Osteoblast dysfunction is a multifactorial process caused both by decreased osteoblast stimulation and increased osteoblast inhibition. Serum levels of insulin-like growth factor-1 (IGF-1), an osteoblast trophic factor, are lower in cirrhotics compared to controls[19]. Supplementation of IGF-1 in cirrhotic rats results in improvement in bone mass and bone density[20]. Vitamin K is also involved in bone metabolism through carboxylation of the non-collagenous bone protein, osteocalcin, and has been shown to stimulate osteoblastogenesis and inhibit osteoclastogenesis[21,22]. Vitamin K levels may be decreased in patients with severe cholestasis and impaired fatty-soluble vitamin absorption resulting in impaired osteoblast function. Indeed a meta-analysis does indicate some reduction in bone loss with vitamin K supplementation, but was not performed exclusively in patients with chronic liver disease[23].

Elevated levels of bilirubin, bile salts, and altered fibronectin production may also play a role in decreased bone formation through osteoblast inhibition in PBC. In one study, plasma mitogenic activity of osteoblasts was significantly lower in patients with cholestatic liver disease compared to healthy controls[24]. In addition, removal of bilirubin by plasma photobleaching resulted in improved plasma mitogenic activity[24]. Similarly, elevated lithocolic acid concentrations have been shown to decrease osteoblast survival through impaired

vitamin D stimulation of osteoblast gene transcription[25]. Patients with chronic liver disease also exhibit altered hepatic fibronectin production, resulting in increased production of a fibronectin isoform containing the oncofetal domain, which inhibits osteoblast-mediated mineralization in humans and mice[26].

Increased bone resorption may also play a role in osteoporosis in PBC in certain populations such as post-menopausal women and men with hypogonadism[5,14,16]. One study showed increased osteoblast numbers and increased eroded surface area indicative of increased resorption, but only in female patients with cholestatic liver disease[16]. Estrogen promotes apoptosis of osteoclasts and its absence results in a sharp decline in bone mineral density (BMD) after menopause[27,28]. Men with cholestatic liver disease show no signs of increased bone resorption despite similar degrees of osteopenia[16].

Theoretically, calcium and vitamin D deficiencies may develop in those with cholestatic liver disease leading to secondary hyperparathyroidism and increased bone resorption. Data is conflicting, however. Some studies have found decreased calcium absorption and serum vitamin D levels in PBC patients compared to controls[5,29], but others have found normal vitamin D, calcium, and PTH levels even among osteoporotic patients with PBC[16,17,26]. In addition, vitamin D supplementation to normal levels has not been shown to improve BMD in PBC[30,31]. While vitamin D absorption may be impaired in those taking cholestyramine, this is overcome by increasing the oral dose of vitamin D[32].

Diagnosis and monitoring
No data exists as to the optimal timing of screening and monitoring for osteoporosis in PBC, however, expert opinion recommends bone densitometry be performed in all PBC patients at diagnosis[11]. The World Health Organization defines osteoporosis as BMD at the spine or proximal femur less than 2.5 standard deviations (SDs) below the mean of a young adult population (expressed as a T score)[33]. Osteopenia refers to those with a T score between -1.0 and -2.5 SDs below the mean. Patients who initially have normal bone densitometry should be reassessed every 2-3 years with repeat bone densitometry, while those with additional risk factors for low bone density and fractures (*i.e.*, severe cholestasis, long-term corticosteroid use, postmenopausal women, BMI < 19, menopause before age 45, alcohol abuse, smoking) should be reassessed annually. Serum calcium, phosphorus, 25-vitamin D, and parathyroid hormone levels should also be checked at diagnosis of PBC and yearly thereafter[14,34,35]. Patients in whom treatment has been initiated should have repeat BMD measured every 1-2 years[36].

MANAGEMENT
Prevention
General measures to prevent bone loss in PBC are extrapolated from osteoporosis risk factors in the general population. Epidemiologic data suggests lifestyle factors such as tobacco and alcohol use, low dietary calcium intake, and low levels of exercise are associated with decreased bone density and fracture risk[33]. Based on this, tobacco and alcohol cessation are recommended in all PBC patients to reduce risk of bone loss. A balanced diet with adequate levels of calcium and vitamin D should also be encouraged. General recommendations for daily dietary calcium and vitamin D intake in at-risk populations (women older than 50) are 1200 mg and 800 IU daily, respectively[33].

Evidence for routine weight-bearing exercise comes from menopausal women. In a 16-year prospective study of early-postmenopausal women with mild oste-openia, those who exercised regularly had significant improvement in BMD compared to controls. This finding was sustained and even greater at 16 years compared to 4[37].

Treatment
Timing of treatment is based on recommendations from the postmenopausal osteoporosis literature[36]. Treatment should be initiated in all PBC patients with osteoporosis (T score < -2.5). The ideal time to start treatment in those with osteopenia (T score < -1 to -2.5) is less well established. The National Osteoporosis Foundation recommends treatment in individuals with a prior hip or vertebral fracture or 10-year hip fracture risk ≥ 3% or osteoporosis-related fracture risk ≥ 20% based on the World Health Organization Fracture Risk Assessment Tool in those with osteopenia[36]. In PBC, patients with a T score < 1.5 appear to be at increased risk of fracture[3] and initiation of specific treatment could be considered at this point (Figure 1). A number of different therapeutic approaches have been evaluated, however, most are limited by small sample size and short follow-up (Tables 1 and 2). The duration of treatment is unclear even in the postmenopausal population, though the National Osteoporosis Foundation emphasizes that treatment should not be indefinite and generally is continued for anywhere from 2 to 5 years based on individual risk assessment[36].

THERAPEUTIC OPTIONS
Vitamin D and calcium supplementation
In PBC patients with osteopenia or osteoporosis, vitamin D and calcium supplementation has not been shown to improve BMD or reduce fracture risk, though given few side effects and potential for deficiency, supplementation is generally recommended[14,34,35]. The only randomized controlled trial (RCT) looking at calcitriol supplementation did not find a significant improvement in BMD from baseline at one year, though BMD did significantly worsen in those who received no treatment[38]. Most studies use some combination of calcium and vitamin D supplementation as standard of care in both the intervention and control groups, though

Table 1 Summary of results in randomized-controlled trials of active agent *vs* placebo or no treatment

Agent	Treatment	No. of patients (*n*)	BMD changes at 1 yr (%)	BMD changes at 2 yr (%)	Fractures (*n*)
Etidronate	400 mg/d (3 mo cycles)	6 (etidronate)	+1.0 (L), +0.2 (F)	N/A	0
Wolfhagen *et al*[43], 1997		6 (no treatment)[3]	-1.7 (L), +0.4 (F)		0
Lindor *et al*[6], 2000	400 mg/d (3 mo cycles)	29 (etidronate)	+0.7 (L), +1.3 (F)	+1.0 (L), +0.5 (F)	4 (V)
		31 (placebo)	-0.6 (L), +0.9 (F)	+2.6 (L), +0.8 (F)	4 (V)
Alendronate	70 mg/wk	15 (alendronate)	+10.4 (L)[1,2], +1.4 (F)[1]	N/A	1 (V), 0 (P)
Zein *et al*[44], 2005		13 (placebo)	-0.1 (L)[1], -2.1 (F)[1]		0 (V), 1 (P)
HRT	50 mcg twice weekly TD	8 (HRT)	+3.1 (L)[2], +1.7 (F)[1,2]	N/A	0
Ormarsdottir *et al*[51], 2004	estradiol + 2.5 mg/d progestin	9 (no treatment)[3]	+1.0 (L), -0.6 (F)[1]		0
Boone *et al*[9], 2006	0.05 mg/d TD estradiol + 0.25	8 (HRT)	N/A	-0.6 (L), +0.2 (F)	0 (V)
	mg/d TD progestin	14 (placebo)		-0.8 (L), -3.7 (F)[2]	2 (V)
Sodium fluoride	50 mg/d sodium fluoride	8 (fluoride)	N/A	+2.9 (L)[1]	0
Guañabens *et al*[7], 1992		8 (placebo)		-6.6 (L)[1,2]	0
Calcitriol	0.5 mcg/d BID calcitriol	17 (calcitriol)	+0.1 (L)[1]	N/A	N/A
Shiomi *et al*[38], 1999		17 (no treatment)[3]	-3.1 (L)[1]		
Vitamin K	45 mg/d vitamin K$_2$	15 (vitamin K)	+0.3 (L)[1]	-0.8 (L)[1]	N/A
Nishiguchi *et al*[57], 2001		15 (no treatment)[3]	-3.5 (L)[1]	-6.9 (L)[1]	

[1]Statistical significance ($P < 0.05$) between groups; [2]Statistical difference ($P < 0.05$) from baseline; [3]Patients in no treatment groups received vitamin D and calcium supplementation. L: Lumbar; F: Femoral; V: Vertebral; P: Peripheral; BMD: Bone mineral density.
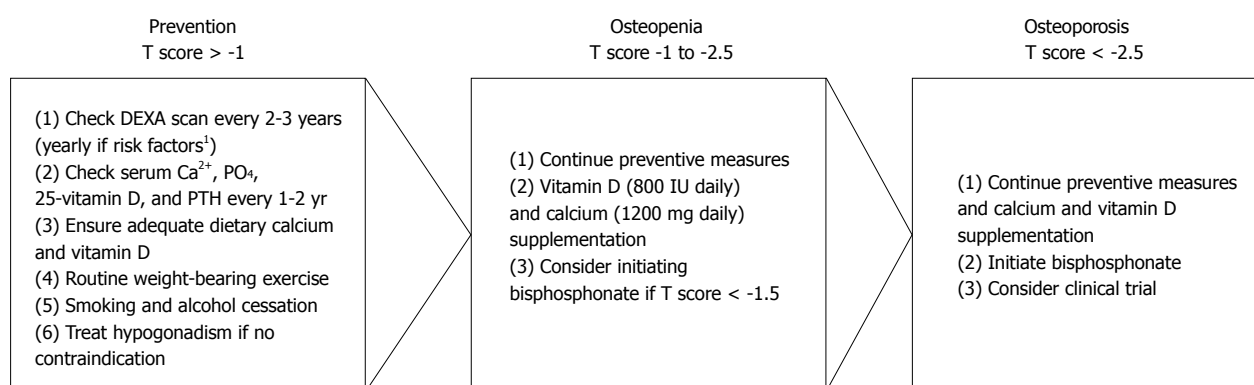

Figure 1 Summary of prevention and treatment strategies for osteoporosis in Primary biliary cholangitis. [1]Prolonged steroid use (> 3 mo), BMI < 19 kg/m^2, heavy alcohol or tobacco use.

notably, BMD continues to worsen in the control group despite supplementation[9,39,40].

Bisphosphonates

Bisphosphonates reduce bone resorption and are effective in increasing BMD while reducing fractures in postmenopausal osteoporosis[41]. Their effectiveness in PBC is not as clear due to the small number of studies with small study size and short follow-up. A 2011 Cochrane review of bisphosphonates in PBC concluded there was insufficient data supporting improved BMD and fracture risk[42]. It appears etidronate is not effective in improving BMD and preventing fractures in PBC. In two RCTs comparing etidronate to calcium alone or placebo, neither demonstrated an improvement in BMD from baseline with etidronate[6,43], though one did show significantly less bone loss compared to calcium alone[43]. Neither showed a difference in fracture risk over 1-2 years of follow-up[6,43].

Alendronate, a nitrogen-containing bisphosphonate as opposed to etidronate, has had more promising results in PBC. Only one RCT has been performed comparing alendronate to placebo in PBC[44]. In this study, 34 patients were randomized to receive oral alendronate or placebo. After 1 year, patients in the alendronate arm experienced a 10.4% increase in lumbar spine BMD compared to -0.12% for placebo[44]. However, there was no difference in fracture incidence over a year and the study included only one male and two post-menopausal females[44].

Several studies have also compared bisphosphonates to one another. Alendronate was found to be superior to etidronate in terms of BMD improvement in a small trial of 32 women with PBC and osteopenia in which patients were randomized to receive cyclical etidronate or weekly alendronate[45]. The alendronate group had significantly higher lumbar BMD (5.8%) at the end of 2 years compared to the etidronate group (1.9%). There was no significant improvement in either lumbar or femoral BMD in the etidronate group from baseline. However, 2 patients in the alendronate group experienced new non-vertebral fractures during the study period compared to 1 in the etidronate group[45].

Ibandronate was also compared to alendronate in

Table 2 Summary of results in comparative randomized controlled trials of different agents for treatment of osteoporosis in primary biliary cholangitis

Agent	Treatment	No. of patients (n)	BMD changes at 1 yr (%)	BMD changes at 2 yr (%)	Fractures (n)
Guañabens et al[39], 1997					
Etidronate	400 mg/d (3 mo cycles)	13 (etidronate)	-0.1 (L), -0.4 (F)	+0.5 (L)[2], -0.2 (F)	0 (V), 3 (P)
Sodium fluoride	50 mg/d sodium fluoride	10 (fluoride)	-1.7 (L), -0.6 (F)	-2.1 (L), -1.5 (F)	2 (V), 2 (P)
Guañabens et al[45], 2003					
Alendronate	10 mg/d	13 (alendronate)		+5.8 (L)[1,2], +3.9 (F)[1,2]	0 (V), 2 (P)
Etidronate	400 mg/d (3 mo cycles)	13 (etidronate)		+1.9 (L)[1], +0.4 (F)[1]	0 (V), 1 (P)
Guañabens et al[2], 2005					
Alendronate	10 mg/d	16 (alendronate)	+3.3 (L)[2], +1.2 (F)[2]		
Alendronate	70 mg/wk	10 (alendronate)	+1.2 (L), -0.3 (F)		
Guañabens et al[46], 2013					
Ibandronate	150 mg/mo	14 (ibandronate)	+3.8 (L), +1.0 (F)	+5.7 (L)[2], +1.1 (F)	0 (V), 0 (P)
Alendronate	70 mg/wk	19 (alendronate)	+4.6 (L), +1.4 (F)	+4.5 (L)[2], +2.5 (F)	1 (V), 0 (P)

[1]Statistical significance ($P < 0.05$) between groups; [2]Statistical difference ($P < 0.05$) from baseline. L: Lumbar; F: Femoral; V: Vertebral; P: Peripheral; BMD: Bone mineral density.

a more recent study of 42 post-menopausal women with PBC and osteoporosis[46]. In this study, women were randomized to receive IV ibandronate monthly or weekly oral alendronate. There was no significant difference in BMD between the 2 groups at the end of 2 years, however, compliance was significantly better in the ibandronate group[46]. One patient in the alendronate group developed a new vertebral fracture. Another advantage of IV administration is the avoidance of the theoretical concern of esophagitis and variceal bleeding with oral bisphosphonates in cirrhotics. However, none of the studies with alendronate and etidronate noted any esophagitis or variceal bleeds[6,40,43-46].

Evidence for the use of bisphosphonates in PBC is limited with only one small trial of alendronate showing improvement in BMD compared to placebo, though this occurred almost exclusively in postmenopausal women, and none demonstrating fracture risk reduction[6,43,44]. Similarly powered trials in postmenopausal osteoporosis have shown improvement benefit in terms of both fracture reduction and BMD improvement[47], and the PBC-specific benefit of bisphosphonates remains unclear.

Hormone replacement
Estrogens also have strong anti-resorptive effect on bones and for a period of time were widely used in postmenopausal osteoporosis[48]. Concerns over worsening of cholestasis initially limited their use in PBC. However, several observational studies did not show any significant worsening in liver disease with hormone replacement therapy (HRT)[49,50] and subsequently two RCTs similarly found no worsening in liver disease[9,51]. In these RCTs, those randomized to transdermal estrogen and progesterone had femoral and vertebral BMD compared to controls after 1-2 years of treatment[9,51], though only one showed an improvement in BMD from baseline[51] and neither showed a reduction in fracture risk[9,51]. In addition, both studies noted an increase in noncholestatic adverse events, such as vaginal bleeding and headaches, in the HRT arm leading to increased

dropout[9,51]. Hormone replacement is also associated with increased risk of venous thromboembolism, stroke, ischemic heart disease, and breast cancer and is not recommended in women older than age 60 or greater than 10 years after menopause[52]. Hormone replacement may be effective in improving BMD in PBC, though no improvement in fracture risk has been shown and side effects limit their use.

Calcitonin
Calcitonin has been shown to have some effect in postmenopausal osteoporosis, however, data in PBC is lacking. A crossover study of IV calcitonin for 6 mo compared to oral calcium supplementation did not find a significant difference between the two and BMD ultimately fell in both groups[53]. A 3-year study of vitamin D, calcium, and IM calcitonin found significantly less BMD loss in treatment patients compared to controls who received no treatment[54,55]. However, there was no significant improvement in the calcitonin group and, since the control group received no therapy, the stabilization of BMD may have been from calcium and vitamin D supplementation[55].

Selective estrogen receptor modulators
Raloxifene is a selective estrogen receptor modulator that maintains the antiresorptive effects of estrogen in bone with anti-estrogen effects in the uterus and breast. A single pilot study has examined the efficacy of raloxifene in PBC. In this study, 9 women with PBC treated with raloxifene were compared to 3 age-matched controls[56]. After 1 year, there was a small (0.02 g/cm^2 vs 0.00 g/cm^2) but significant improvement in lumbar BMD compared to controls, though there was no difference in femoral BMD and no data on fracture risk[56].

Sodium fluoride
Sodium fluoride increases bone formation and has been shown to be effective in postmenopausal osteoporosis,

but is not as effective as anti-resorptive agents. One randomized, controlled trial examined sodium fluoride in PBC and found a significant increase in BMD (+2.9%) compared to placebo (-6.6%) in 22 women with PBC followed for 2 years[7]. No fractures occurred in either group[7]. A subsequent trial comparing sodium fluoride to etidronate, however, found etidronate to be superior and actually observed a decrease in femoral BMD in the sodium fluoride group after 2 years[39]. In addition, 2 patients in the sodium fluoride group experienced new vertebral fractures compared to none in the etidronate group[39].

Vitamin K
Patients with severe cholestasis may have decreased absorption of fat-soluble vitamins, such as vitamin K. Vitamin K is involved in bone formation through stimulation of osteoblastogenesis[21,22]. One meta-analysis did indicate some reduction in bone loss with vitamin K supplementation, but did not focus on patients with cholestatic liver disease[23]. One small RCT in PBC found significantly less bone loss in vitamin K patients compared to controls, but was ultimately not effective in improving BMD[57].

Parathyroid hormone
Human parathyroid hormone (PTH) improves BMD and reduces fractures in postmenopausal osteoporosis through stimulating bone formation, the major driver of osteoporosis in PBC[58,59]. The recombinant form consisting of the bioactive portion of the hormone (teriparatide) is approved for treatment of postmenopausal osteoporosis, but has not been studied in PBC. Recombinant human PTH 1-34 (rhPTH 1-34) has been studied in rats that have undergone biliary ductal ligation[60]. In this study, rats that underwent biliary ductal ligation had significant worsening in BMD compared to rats that underwent a sham operation. Biliary ductal ligation rats were then administered rhPTH 1-34 at 40 and 80 µg/kg/d. Those rats who received 40 µg/kg/d experienced significant improvement in femoral and tibial BMD compared to untreated rats and those who received 80 µg/kg/d (who did not experience significant improvement compared to untreated rats)[60]. No trials have been undertaken in humans with PBC.

PBC-specific therapies
Therapies directed at PBC itself have not been shown to improve bone disease. Patients enrolled in a randomized, controlled trial of ursodeoxycholic acid (UDCA) were followed over a 3-year period with dual-photon densitometry annually. After 3 years, there was no significant difference in lumbar BMD between UDCA and placebo[61].

In the initial phase 3 trial of obeticholic acid, BMD was measured at baseline and 12 mo[62]. BMD continued to decline in the obeticholic acid groups (both 5-10 mg and 10 mg groups), however, this decline was significantly less at the femoral neck compared to placebo. BMD

decreased in both groups at the lumbar spine as well and, while there was a trend toward less decline in the obeticholic acid groups, there was no significant difference[62]. There was no difference in fracture rates[62].

No data currently exists evaluating the effects of fibrates on BMD when used to treat PBC.

Future therapies
Concerns over long-term efficacy and safety of bisphosphonate use in postmenopausal osteoporosis have led to ongoing development of new medications. Abaloparatide, a parathyroid hormone-related peptide analog, was recently approved by the Food and Drug Administration for treatment of postmenopausal osteoporosis and may avoid the pro-resorptive and hypercalcemic effects of teriparatide[63]. Along with teriparatide, these recombinant human PTH agents differ in that they promote bone formation, the primary deficit in PBC, as opposed to decrease resorption, the driving issue in postmenopausal osteoporosis and the current target of most treatments. Based on our understanding of the pathogenesis of osteoporosis in PBC, future studies should include therapies that promote bone formation and ample male and premenopausal female PBC patients to decrease the likelihood that results reflect only treatment of postmenopausal osteoporosis.

CONCLUSION
Osteoporosis is a common complication of PBC resulting in a significantly increased risk of fractures especially in those with a T score < -1.5. Severity of bone disease is related to the severity and duration of the underlying liver disease as well as increasing age. The mechanism of decreased bone density primarily involves decreased bone formation resulting from decreased osteoblast function. Increased bone resorption may also play a role in postmenopausal women.

For prevention of osteoporosis, mitigation of risk factors is recommended through smoking and alcohol cessation as well as a balanced diet and regular weight-bearing exercise. Treatment is recommended in patients who have experienced fractures or have osteoporosis (T score < -2.5), though may also be considered in those with a T score < -1.5 in PBC. No treatment has been adequately shown to reduce fractures in PBC, though the bisphosphonates ibandronate and alendronate may be effective in increasing BMD. HRT may also be effective in improving BMD in PBC, though with more side effects. Treatment of the underlying liver disease with UDCA or OCA does not appear to effectively treat the bone disease. Ultimately further research is required, with special attention to anabolic bone agents, to identify effective treatment in PBC-related osteoporosis.

REFERENCES
1   **Newton J**, Francis R, Prince M, James O, Bassendine M, Rawlings D, Jones D. Osteoporosis in primary biliary cirrhosis revisited. *Gut*

2001; **49**: 282-287 [PMID: 11454807 DOI: 10.1136/gut.49.2.282]

2   **Guañabens N**, Parés A, Ros I, Caballería L, Pons F, Vidal S, Monegal A, Peris P, Rodés J. Severity of cholestasis and advanced histological stage but not menopausal status are the major risk factors for osteoporosis in primary biliary cirrhosis. *J Hepatol* 2005; **42**: 573-577 [PMID: 15763344 DOI: 10.1016/j.jhep.2004.11.035]

3   **Guañabens N**, Cerdá D, Monegal A, Pons F, Caballería L, Peris P, Parés A. Low bone mass and severity of cholestasis affect fracture risk in patients with primary biliary cirrhosis. *Gastroenterology* 2010; **138**: 2348-2356 [PMID: 20178794 DOI: 10.1053/j.gastro.2010.02.016]

4   **Guichelaar MM**, Kendall R, Malinchoc M, Hay JE. Bone mineral density before and after OLT: long-term follow-up and predictive factors. *Liver Transpl* 2006; **12**: 1390-1402 [PMID: 16933236 DOI: 10.1002/lt.20874]

5   **Guañabens N**, Parés A, Mariñoso L, Brancós MA, Piera C, Serrano S, Rivera F, Rodés J. Factors influencing the development of metabolic bone disease in primary biliary cirrhosis. *Am J Gastroenterol* 1990; **85**: 1356-1362 [PMID: 2220729]

6   **Lindor KD**, Jorgensen RA, Tiegs RD, Khosla S, Dickson ER. Etidronate for osteoporosis in primary biliary cirrhosis: a randomized trial. *J Hepatol* 2000; **33**: 878-882 [PMID: 11131448 DOI: 10.1016/S0168-8278(00)80118-X]

7   **Guañabens N**, Parés A, del Rio L, Roca M, Gómez R, Muñoz J, Rodés J. Sodium fluoride prevents bone loss in primary biliary cirrhosis. *J Hepatol* 1992; **15**: 345-349 [PMID: 1447501]

8   **Guañabens N**, Parés A, Navasa M, Martínez de Osaba MJ, Hernández ME, Muñoz J, Rodés J. Cyclosporin A increases the biochemical markers of bone remodeling in primary biliary cirrhosis. *J Hepatol* 1994; **21**: 24-28 [PMID: 7963418]

9   **Boone RH**, Cheung AM, Girlan LM, Heathcote EJ. Osteoporosis in primary biliary cirrhosis: a randomized trial of the efficacy and feasibility of estrogen/progestin. *Dig Dis Sci* 2006; **51**: 1103-1112 [PMID: 16865577 DOI: 10.1007/s10620-006-8015-x]

10   **Cohen SM**, Te HS, Levitsky J. Operative risk of total hip and knee arthroplasty in cirrhotic patients. *J Arthroplasty* 2005; **20**: 460-466 [PMID: 16124961 DOI: 10.1016/j.arth.2004.05.004]

11   **Lindor KD**, Gershwin ME, Poupon R, Kaplan M, Bergasa NV, Heathcote EJ; American Association for Study of Liver Diseases. Primary biliary cirrhosis. *Hepatology* 2009; **50**: 291-308 [PMID: 19554543 DOI: 10.1002/hep.22906]

12   **Long RG**, Varghese Z, Meinhard EA, Skinner RK, Wills MR, Sherlock S. Parenteral 1,25-dihydroxycholecalciferol in hepatic osteomalacia. *Br Med J* 1978; **1**: 75-77 [PMID: 620204]

13   **Compston JE**, Crowe JP, Horton LW. Treatment of osteomalacia associated with primary biliary cirrhosis with oral 1-alpha-hydroxy vitamin D3. *Br Med J* 1979; **2**: 309 [PMID: 476441]

14   **Bernstein CN**, Leslie WD, Leboff MS. AGA technical review on osteoporosis in gastrointestinal diseases. *Gastroenterology* 2003; **124**: 795-841 [PMID: 12612917 DOI: 10.1016/S0016-5085(03)01062-X]

15   **Hay JE**. Bone disease in cholestatic liver disease. *Gastroenterology* 1995; **108**: 276-283 [PMID: 7806050]

16   **Guichelaar MM**, Malinchoc M, Sibonga J, Clarke BL, Hay JE. Bone metabolism in advanced cholestatic liver disease: analysis by bone histomorphometry. *Hepatology* 2002; **36**: 895-903 [PMID: 12297836 DOI: 10.1053/jhep.2002.36357]

17   **Hodgson SF**, Dickson ER, Wahner HW, Johnson KA, Mann KG, Riggs BL. Bone loss and reduced osteoblast function in primary biliary cirrhosis. *Ann Intern Med* 1985; **103**: 855-860 [PMID: 4062087]

18   **Ackerman Z**, Weinreb M, Amir G, Pollak RD. Bone mineral metabolism and histomorphometry in rats with cholestatic liver disease. *Liver* 2002; **22**: 166-172 [PMID: 12028412]

19   **Gallego-Rojo FJ**, Gonzalez-Calvin JL, Muñoz-Torres M, Mundi JL, Fernandez-Perez R, Rodrigo-Moreno D. Bone mineral density, serum insulin-like growth factor I, and bone turnover markers in viral cirrhosis. *Hepatology* 1998; **28**: 695-699 [PMID: 9731561 DOI: 10.1002/hep.510280315]

20   **Cemborain A**, Castilla-Cortázar I, García M, Quiroga J, Muguerza

B, Picardi A, Santidrián S, Prieto J. Osteopenia in rats with liver cirrhosis: beneficial effects of IGF-I treatment. *J Hepatol* 1998; **28**: 122-131 [PMID: 9537849 DOI: 10.1016/S0168-8278(98)80211-0]

21   **Houben RJ**, Rijkers DT, Stanley TB, Acher F, Azerad R, Käkönen SM, Vermeer C, Soute BA. Characteristics and composition of the vitamin K-dependent gamma-glutamyl carboxylase-binding domain on osteocalcin. *Biochem J* 2002; **364**: 323-328 [PMID: 11988107]

22   **Koshihara Y**, Hoshi K, Okawara R, Ishibashi H, Yamamoto S. Vitamin K stimulates osteoblastogenesis and inhibits osteoclastogenesis in human bone marrow cell culture. *J Endocrinol* 2003; **176**: 339-348 [PMID: 12630919 DOI: 10.1677/joe.0.1760339]

23   **Cockayne S**, Adamson J, Lanham-New S, Shearer MJ, Gilbody S, Torgerson DJ. Vitamin K and the prevention of fractures: systematic review and meta-analysis of randomized controlled trials. *Arch Intern Med* 2006; **166**: 1256-1261 [PMID: 16801507 DOI: 10.1001/archinte.166.12.1256]

24   **Janes CH**, Dickson ER, Okazaki R, Bonde S, McDonagh AF, Riggs BL. Role of hyperbilirubinemia in the impairment of osteoblast proliferation associated with cholestatic jaundice. *J Clin Invest* 1995; **95**: 2581-2586 [PMID: 7769100 DOI: 10.1172/JCI117959]

25   **Ruiz-Gaspà S**, Guañabens N, Enjuanes A, Peris P, Martinez-Ferrer A, de Osaba MJ, Gonzalez B, Alvarez L, Monegal A, Combalia A, Parés A. Lithocholic acid downregulates vitamin D effects in human osteoblasts. *Eur J Clin Invest* 2010; **40**: 25-34 [PMID: 20055894 DOI: 10.1111/j.1365-2362.2009.02230.x]

26   **Kawelke N**, Bentmann A, Hackl N, Hager HD, Feick P, Geursen A, Singer MV, Nakchbandi IA. Isoform of fibronectin mediates bone loss in patients with primary biliary cirrhosis by suppressing bone formation. *J Bone Miner Res* 2008; **23**: 1278-1286 [PMID: 18348696 DOI: 10.1359/jbmr.080313]

27   **Hughes DE**, Dai A, Tiffee JC, Li HH, Mundy GR, Boyce BF. Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-beta. *Nat Med* 1996; **2**: 1132-1136 [PMID: 8837613 DOI: 10.1038/nm1096-1132]

28   **Armas LA**, Recker RR. Pathophysiology of osteoporosis: new mechanistic insights. *Endocrinol Metab Clin North Am* 2012; **41**: 475-486 [PMID: 22877425 DOI: 10.1016/j.ecl.2012.04.006]

29   **Bengoa JM**, Sitrin MD, Meredith S, Kelly SE, Shah N, Baker AL, Rosenberg IH. Intestinal calcium absorption and vitamin D status in chronic cholestatic liver disease. *Hepatology* 1984; **4**: 261-265 [PMID: 6706300]

30   **Herlong HF**, Recker RR, Maddrey WC. Bone disease in primary biliary cirrhosis: histologic features and response to 25-hydroxyvitamin D. *Gastroenterology* 1982; **83**: 103-108 [PMID: 6978827]

31   **Matloff DS**, Kaplan MM, Neer RM, Goldberg MJ, Bitman W, Wolfe HJ. Osteoporosis in primary biliary cirrhosis: effects of 25-hydroxyvitamin D3 treatment. *Gastroenterology* 1982; **83**: 97-102 [PMID: 7075948]

32   **Compston JE**, Thompson RP. Intestinal absorption of 25-hydroxyvitamin D and osteomalacia in primary biliary cirrhosis. *Lancet* 1977; **1**: 721-724 [PMID: 66519]

33   **Office of the Surgeon General (US)**. Bone Health and Osteoporosis: A Report of the Surgeon General. *US Heal Hum Serv* (Internet) 2004; **437** [PMID: 20945569 DOI: 10.2165/00002018-200932030-00004]

34   **Parés A**, Guañabens N. Osteoporosis in primary biliary cirrhosis: pathogenesis and treatment. *Clin Liver Dis* 2008; **12**: 407-24 [PMID: 18456188 DOI: 10.1016/j.cld.2008.02.005]

35   **Glass LM**, Su GL. Metabolic Bone Disease in Primary Biliary Cirrhosis. *Gastroenterol Clin North Am* 2016; **45**: 333-343 [PMID: 27261902 DOI: 10.1016/j.gtc.2016.02.009]

36   **Cosman F**, de Beur SJ, LeBoff MS, Lewiecki EM, Tanner B, Randall S, Lindsay R; National Osteoporosis Foundation. Clinician's Guide to Prevention and Treatment of Osteoporosis. *Osteoporos Int* 2014; **25**: 2359-2381 [PMID: 25182228 DOI: 10.1007/s00198-014-2794-2]

37   **Kemmler W**, Engelke K, von Stengel S. Long-Term Exercise and Bone Mineral Density Changes in Postmenopausal Women--Are There Periods of Reduced Effectiveness? *J Bone Miner Res* 2016;

31: 215-222 [PMID: 26234411 DOI: 10.1002/jbmr.2608]

38   **Shiomi S**, Masaki K, Habu D, Takeda T, Nishiguchi S, Kuroki T, Ochi H. Calcitriol for bone loss in patients with primary biliary cirrhosis. *J Gastroenterol* 1999; **34**: 241-245 [PMID: 10213125]

39   **Guañabens N**, Parés A, Monegal A, Peris P, Pons F, Alvarez L, de Osaba MJ, Roca M, Torra M, Rodés J. Etidronate versus fluoride for treatment of osteopenia in primary biliary cirrhosis: preliminary results after 2 years. *Gastroenterology* 1997; **113**: 219-224 [PMID: 9207281 DOI: 10.1016/S0016-5085(97)70098-2]

40   **Musialik J**, Petelenz M, Gonciarz Z. Effects of alendronate on bone mass in patients with primary biliary cirrhosis and osteoporosis: preliminary results after one year. *Scand J Gastroenterol* 2005; **40**: 873-874 [PMID: 16109666 DOI: 10.1080/00365520510023279]

41   **Sanderson J**, Martyn-St James M, Stevens J, Goka E, Wong R, Campbell F, Selby P, Gittoes N, Davis S. Clinical effectiveness of bisphosphonates for the prevention of fragility fractures: A systematic review and network meta-analysis. *Bone* 2016; **89**: 52-58 [PMID: 27262775 DOI: 10.1016/j.bone.2016.05.013]

42   **Rudic JS**, Giljaca V, Krstic MN, Bjelakovic G, Gluud C. Bisphosphonates for osteoporosis in primary biliary cirrhosis. *Cochrane Database Syst Rev* 2011; CD009144 [PMID: 22161446 DOI: 10.1002/14651858.CD009144.pub2]

43   **Wolfhagen FH**, van Buuren HR, den Ouden JW, Hop WC, van Leeuwen JP, Schalm SW, Pols HA. Cyclical etidronate in the prevention of bone loss in corticosteroid-treated primary biliary cirrhosis. A prospective, controlled pilot study. *J Hepatol* 1997; **26**: 325-330 [PMID: 9059953]

44   **Zein CO**, Jorgensen RA, Clarke B, Wenger DE, Keach JC, Angulo P, Lindor KD. Alendronate improves bone mineral density in primary biliary cirrhosis: a randomized placebo-controlled trial. *Hepatology* 2005; **42**: 762-771 [PMID: 16175618 DOI: 10.1002/hep.20866]

45   **Guañabens N**, Parés A, Ros I, Alvarez L, Pons F, Caballería L, Monegal A, Martínez de Osaba MJ, Roca M, Peris P, Rodés J. Alendronate is more effective than etidronate for increasing bone mass in osteopenic patients with primary biliary cirrhosis. *Am J Gastroenterol* 2003; **98**: 2268-2274 [PMID: 14572578 DOI: 10.1016/S0002-9270(03)00550-1]

46   **Guañabens N**, Monegal A, Cerdá D, Muxí Á, Gifre L, Peris P, Parés A. Randomized trial comparing monthly ibandronate and weekly alendronate for osteoporosis in patients with primary biliary cirrhosis. *Hepatology* 2013; **58**: 2070-2078 [PMID: 23686738 DOI: 10.1002/hep.26466]

47   **El Dareer SM**, Tillery KF, Hill DL. Disposition of 5-methyltetrahydrohomofolate in mice, dogs, and monkeys. *Cancer Treat Rep* 1979; **63**: 201-207 [PMID: 109197 DOI: 10.1056/NEJM199005033221803]

48   **Hillard TC**, Whitcroft SJ, Marsh MS, Ellerington MC, Lees B, Whitehead MI, Stevenson JC. Long-term effects of transdermal and oral hormone replacement therapy on postmenopausal bone loss. *Osteoporos Int* 1994; **4**: 341-348 [PMID: 7696830]

49   **Olsson R**, Mattsson LA, Obrant K, Mellström D. Estrogen-progestogen therapy for low bone mineral density in primary biliary cirrhosis. *Liver* 1999; **19**: 188-192 [PMID: 10395037 DOI: 10.1111/j.1478-3231.1999.tb00034.x]

50   **Menon KV**, Angulo P, Boe GM, Lindor KD. Safety and efficacy of estrogen therapy in preventing bone loss in primary biliary cirrhosis. *Am J Gastroenterol* 2003; **98**: 889-892 [PMID: 12738473 DOI: 10.1111/j.1572-0241.2003.07341.x]

51   **Ormarsdóttir S**, Mallmin H, Naessén T, Petrén-Mallmin M, Broomé U, Hultcrantz R, Lööf L. An open, randomized, controlled study of transdermal hormone replacement therapy on the rate of bone loss in primary biliary cirrhosis. *J Intern Med* 2004; **256**: 63-69 [PMID: 15189367 DOI: 10.1111/j.1365-2796.2004.01342.x]

52   **de Villiers TJ**, Gass ML, Haines CJ, Hall JE, Lobo RA, Pierroz DD, Rees M. Global Consensus Statement on menopausal hormone therapy. *Maturitas* 2013; **74**: 391-392 [PMID: 23497918 DOI: 10.1016/j.maturitas.2013.02.001]

53   **Camisasca M**, Crosignani A, Battezzati PM, Albisetti W, Grandinetti G, Pietrogrande L, Biffi A, Zuin M, Podda M. Parenteral calcitonin for metabolic bone disease associated with primary biliary cirrhosis. *Hepatology* 1994; **20**: 633-637 [PMID: 8076921]

54   **Floreani A**, Chiaramonte M, Giannini S, Malvasi L, Lodetti MG, Castrignano R, Giacomini A, D'Angelo A, Naccarato R. Longitudinal study on osteodystrophy in primary biliary cirrhosis (PBC) and a pilot study on calcitonin treatment. *J Hepatol* 1991; **12**: 217-223 [PMID: 2051000 DOI: 10.1016/0168-8278(91)90941-4]

55   **Floreani A**, Zappala F, Fries W, Naccarato R, Plebani M, D'Angelo A, Chiaramonte M. A 3-year pilot study with 1,25-dihydroxyvitamin D, calcium, and calcitonin for severe osteodystrophy in primary biliary cirrhosis. *J Clin Gastroenterol* 1997; **24**: 239-244 [PMID: 9252849 DOI: 10.1097/00004836-199706000-00012]

56   **Levy C**, Harnois DM, Angulo P, Jorgensen R, Lindor KD. Raloxifene improves bone mass in osteopenic women with primary biliary cirrhosis: results of a pilot study. *Liver Int* 2005; **25**: 117-121 [PMID: 15698408 DOI: 10.1111/j.1478-3231.2005.01026.x]

57   **Nishiguchi S**, Shimoi S, Kurooka H, Tamori A, Habu D, Takeda T, Kubo S. Randomized pilot trial of vitamin K2 for bone loss in patients with primary biliary cirrhosis. *J Hepatol* 2001; **35**: 543-545 [PMID: 11682046 DOI: 10.1016/S0168-8278(01)00133-7]

58   **Neer RM**, Arnaud CD, Zanchetta JR, Prince R, Gaich GA, Reginster JY, Hodsman AB, Eriksen EF, Ish-Shalom S, Genant HK, Wang O, Mitlak BH. Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. *N Engl J Med* 2001; **344**: 1434-1441 [PMID: 11346808 DOI: 10.1056/NEJM200105103441904]

59   **Miller PD**, Hattersley G, Riis BJ, Williams GC, Lau E, Russo LA, Alexandersen P, Zerbini CA, Hu MY, Harris AG, Fitzpatrick LA, Cosman F, Christiansen C; ACTIVE Study Investigators. Effect of Abaloparatide vs Placebo on New Vertebral Fractures in Postmenopausal Women With Osteoporosis: A Randomized Clinical Trial. *JAMA* 2016; **316**: 722-733 [PMID: 27533157 DOI: 10.1001/jama.2016.11136]

60   **Dresner-Pollak R**, Gabet Y, Steimatzky A, Hamdani G, Bab I, Ackerman Z, Weinreb M. Human parathyroid hormone 1-34 prevents bone loss in experimental biliary cirrhosis in rats. *Gastroenterology* 2008; **134**: 259-267 [PMID: 18061175 DOI: 10.1053/j.gastro.2007.10.025]

61   **Lindor KD**, Janes CH, Crippin JS, Jorgensen RA, Dickson ER. Bone disease in primary biliary cirrhosis: does ursodeoxycholic acid make a difference? *Hepatology* 1995; **21**: 389-392 [PMID: 7843710]

62   **Nevens F**, Andreone P, Mazzella G, Strasser SI, Bowlus C, Invernizzi P, Drenth JP, Pockros PJ, Regula J, Beuers U, Trauner M, Jones DE, Floreani A, Hohenester S, Luketic V, Shiffman M, van Erpecum KJ, Vargas V, Vincent C, Hirschfield GM, Shah H, Hansen B, Lindor KD, Marschall HU, Kowdley KV, Hooshmand-Rad R, Marmon T, Sheeron S, Pencek R, MacConell L, Pruzanski M, Shapiro D; POISE Study Group. A Placebo-Controlled Trial of Obeticholic Acid in Primary Biliary Cholangitis. *N Engl J Med* 2016; **375**: 631-643 [PMID: 27532829 DOI: 10.1056/NEJMoa1509840]

63   **Khosla S**, Hofbauer LC. Osteoporosis treatment: recent developments and ongoing challenges. *Lancet Diabetes Endocrinol* 2017; **5**: 898-907 [PMID: 28689769 DOI: 10.1016/S2213-8587(17)30188-2]

Ubiquitin-proteasome system and oxidative stress in liver transplantation

Norma Alva, Arnau Panisello-Roselló, Marta Flores, Joan Roselló-Catafau, Teresa Carbonell

Norma Alva, Marta Flores, Teresa Carbonell, Department of Cell Biology, Physiology and Immunology, University of Barcelona, Barcelona 08028, Spain

Arnau Panisello-Roselló, Joan Roselló-Catafau, Experimental Pathology Department, Institute of Biomedical Research of Barcelona, Barcelona 08036, Spain

ORCID number: Norma Alva (0000-0001-5039-8270); Arnau Panisello-Roselló (0000-0003-2062-6134); Marta Flores (0000-0002-1062-9676); Joan Roselló-Catafau (0000-0001-7127-4883); Teresa Carbonell (0000-0002-7131-3667).

Abstract

A major issue in organ transplantation is the development of a protocol that can preserve organs under optimal conditions. Damage to organs is commonly a consequence of flow deprivation and oxygen starvation following the restoration of blood flow and reoxygenation. This is known as ischemia-reperfusion injury (IRI): a complex multifactorial process that causes cell damage. While the oxygen deprivation due to ischemia depletes cell energy, subsequent tissue oxygenation due to reperfusion induces many cascades, from reactive oxygen species production to apoptosis initiation. Autophagy has also been identified in the pathogenesis of IRI, although such alterations and their subsequent functional significance are controversial. Moreover, proteasome activation may be a relevant pathophysiological mechanism. Different strategies have been adopted to limit IRI damage, including the supplementation of commercial preservation media with pharmacological agents or additives. In this review, we focus on novel strategies related to the ubiquitin proteasome system and oxidative stress inhibition, which have been used to minimize damage in liver transplantation.

Key words: Liver transplant; Ischemia-reperfusion injury; Oxidative stress; Proteasome; Redox regulation; Ubiquitin

Core tip: Ischemia-reperfusion injury is a complex multifactorial process that causes cell damage during liver transplantation. The role of the ubiquitin proteasome system during liver transplantation remains unclear. The

use of proteasome inhibitors is a new strategy aimed at improving organ preservation.

INTRODUCTION

The definitive treatment option for many liver diseases is liver transplantation; and in these cases, one important issue is the optimization of organ preservation.

During liver transplantation, damage is initiated by vascular occlusion during hepatic resections. This is aggravated by cold storage of the liver graft (usually at 4 ℃) in the preservation solution; and finally by warm reperfusion and subsequent implantation into the recipient. During ischemia, a long period of tissue hypoxia can result in tissue injury and organ dysfunction; as the organ is deprived of oxygen, ATP and energy becomes depleted. Paradoxically, the reintroduction of oxygen during reperfusion aggravates the damage. The whole process has a plethora of consequences that are collectively known as ischemia-reperfusion injury (IRI)[1-3]. While oxygen deprivation due to ischemia depletes cell energy, subsequent reoxygenation due to reperfusion induces many cascades, from induction of reactive oxygen species (ROS)[4] to initiation of apoptosis (Figure 1). Such events are, in part, responsible for organ failure. Variables related to the donor (age, steatosis) and surgery (prolonged ischemia times)[5] are the most commonly reported risk factors for graft dysfunction[6]. Other factors such as shear stress and small graft size[7] have been recognized as important factors associated with oxidative stress, lack of primary function, early dysfunction allograft and biliary complications after liver transplantation[8]. Together with the immunological mechanisms of graft rejection, IRI remains as one of the main clinical problems following organ transplantation.

Current strategies to prevent and modulate IRI include: ischemic pre-[9-15] or post-conditioning[16] protocols ; static cold storage[17] or machine perfusion[18-20] and the use of pharmacological agents[21-23]. The most common methods applied in liver surgery have recently been reviewed[5,24]. Conventional hypothermic cold storage continues to be the main method for liver preservation, largely because of its cost-effectiveness, simplicity and logistics. However, hypothermia can have multiple side effects, including the induction of oxidative stress[25].

Because of the wide range of mechanisms that can contribute to cell damage in IRI (involving ROS, oxidized products, inflammatory mediators and cytokines), adoption of a specific therapeutic strategy often results in only limited improvements in organ transplantation. Given these circumstances, it is worthwhile to focus on the assessment of agents that can counteract the damage induced by oxidative stress. As oxidative stress is the result of an imbalance between the rate of ROS generation and the capacity to detoxify these reactive species[26-28], interventions that could result in ROS scavenging or the detoxifying of ROS products could protect against IRI. Three lines of defence against oxidative stress have been reported[29]. Antioxidant molecules (such as glutathione) represent the first line of antioxidant defence; the second line incorporates enzymatic antioxidants; and the third consists of repair system proteins, including the proteolytic pathways. In this third line, the ubiquitin-proteasome system (UPS) is widely recognized as the main system for degradation of cytosolic proteins[30,31]

The purpose of this review is to present an update of effects of oxidative stress while summarizing recent findings on the role of the UPS in organ preservation and liver transplantation. In this review we outline the current data of the literature, previous search in databases - PubMed, Web of Science and Scopus - that support the hypothesis on the potential involvement of UPS and oxidative stress in IRI. We will focus on the new strategies used to minimize damage in liver transplantation.

STATIC COLD STORAGE, MACHINE PERFUSION AND STORAGE SOLUTIONS

The strategy most commonly adopted to reduce ischemic injury is the cooling of organs and the use of a preservation solution to minimize enzymatic activity and depletion of the energy substrate. Cold storage slows down cellular metabolism[32,33], but this can be responsible for further ATP and energy depletion[34]. In addition, cold has adverse side effects due to the induction of cellular inflammation, alterations of the cytoskeleton[35], and oxidative stress.

Protective strategies to reduce hepatic IRI include the use of the machine perfusion, which represents a new line of research opposed to static cold storage.

Perfusion machine involves a pulsatile perfusion of the liver with a cold (subnormothermic, hypothermic) perfusate[18,36-40]; with normothermic perfusate[41,42]; or with a gradual increase in of the perfusate temperature[43]. These references confirm that machine perfusion protects against IRI damage in animal models. Livers preserved by subnormothermic machine perfusion at 20 ℃ showed significantly less liver damage at the end of reperfusion compared to cold storage. The release of LDH was reduced while the production of bile, ATP levels, glycogen and glutathione content increased in preserved livers by subnormothermic machine perfusion
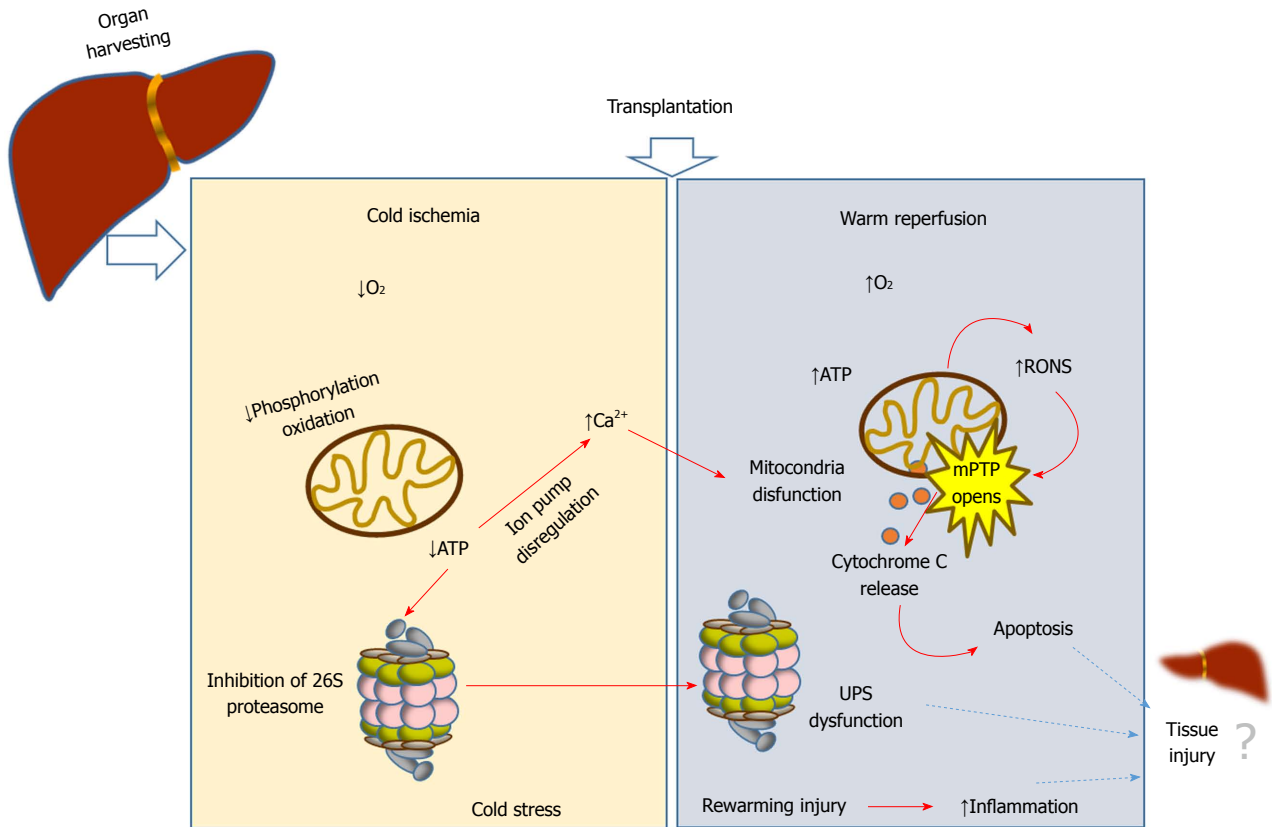
Figure 1 Ischemia-reperfusion injury: A complex multifactorial process that causes cell damage during liver transplantation. While the oxygen deprivation due to ischemia depletes cell energy, subsequent reoxygenation due to reperfusion induces many cascades, from production of reactive oxygen species to initiation of apoptosis. UPS: Ubiquitin-proteasome system; mPTP: mitochondrial permeability transition pore.

than livers submitted to cold storage[44]. Normothermic machine perfusion has also been assessed in discarded human liver grafts[45]. The reported data demonstrate the viability of normothermic perfusion, which results in the continuous production of bile, the fall of lactate levels and the preservation of hepatic morphology. However, the safety and efficacy of machine perfusion is yet to be assessed by randomized controlled clinical trials.

Due to its cost-effectiveness and simplicity, cold storage with conventional hypothermia remains the main method for liver conservation. In fact, major advances in the field of organ preservation have included the development of new improved preservation solutions capable of reducing cold-induced cellular damage[46]. Euro-Collins solution was first developed in the 1970s; while more recently, University of Wisconsin (UW)[47] Celsior[48], Histidine-Ketoglutarate (HTK)[49] and Institute Georges Lopez (IGL-1)[50] solutions tend to be extensively used for liver transplantation. Commercial preservation solutions include oncotic agents, like hydroxy-ethyl starch (HES) in UW and polyethylenglycol-35 (PEG-35) in IGL-1, which confer high viscosity to the media. They also contain metabolic precursors (adenosine and ketoglutarate) and antioxidants (glutathione, allopurinol). Although the use of commercial preservation solutions has improved

conditions for liver graft preservation, with the urgent need to expand the donor pool and the subsequent use of suboptimal grafts, new additives have been proposed to combat oxidant and apoptotic damage with the aim of prolonging graft quality during cold storage.

THE ROLE OF OXIDATIVE STRESS IN IRI

ROS are highly reactive and capable of oxidizing lipids, proteins and DNA[51], thereby leading to structural and cellular changes that may cause oxidative stress and cellular apoptosis. Abundant information has been published demonstrating that increased ROS production is involved in IRI pathology[28,50,52,53]. The involvement of ROS was initially observed based on the detection of enhanced production of chemical products generated by the reaction of ROS with cellular components. Lipid peroxidation products, such as malondialdehyde and hydroxynonenal[13,36,54-56] have been widely used as a biomarkers of oxidative stress in IRI. The accelerated ROS production in post-ischemic tissues has been attributed to enzymes capable of reducing molecular oxygen and forming superoxide: xantine oxidase[57-59], NADPH oxidase[60] and nitric oxide synthase[11]. The contribution of each of these enzymes in IRI is assessed in the excellent review by Granger and Kvietys[28]. All this indicates that radicals can be formed from different

sources, and consequently several protective strategies to decrease liver IRI have targeted different sources of ROS: xanthine oxidase (using allopurinol[59,61]) or NADPH oxidase[62], for example. Other strategies have included pharmacological interventions with antioxidants resulting in the neutralization of ROS effects[63-66].

As mentioned above, Jung et al[29] describe three main lines of defence against oxidative stress. Thus, to those molecules widely recognized as antioxidants, such as glutathione, and enzymatic antioxidants, they add a third line of defence: repair system proteins. This system includes the proteolytic pathways, such as the UPS[30,31]. The activity of the UPS is necessary so that the cells can cope with oxidative stress, but in turn, the activity of the UPS are also modulated by the redox state[67].

THE UBIQUITIN-PROTEASOME SYSTEM

In order to eliminate damaged proteins, cells have highly regulated mechanisms, such as the autophagy-lysosome pathway and the UPS, which is recognized as the principal system for degrading oxidized cytosolic proteins. Proteasomes are protein complexes that, via proteolysis, degrade unnecessary or damaged proteins. It has always appeared that autophagy within lysosomes is involved in the pathogenesis of hepatic IRI, although the specific alterations it causes and their subsequent functional significance are highly controversial[68]. The use of proteasome inhibitors has been demonstrated to enhance myocardial viability[69,70] and protect liver against IRI[4,71-73], which suggests this may be a promising strategy to reduce the damage inherent to transplantation protocols.

Ubiquitin

Ubiquitin is a highly conserved and small 76-amino-acid protein that acts as a post-translational protein modifier and regulates protein lifespan. Ubiquitin was isolated in the 1970s by Goldstein et al[74] and since then has been identified in many cellular processes, including proteasomal proteolysis and also DNA damage repair. Ubiquitin can be attached covalently to a target protein in a process known as ubiquitination. Ubiquitin is conjugated to other proteins through a peptide bond between its C-terminal glycine and a primary amine on the substrate, most typically a lysine residue. Conjugation is dependent on the successive activities of enzymes named E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 (ubiquitin ligase)[75]. In the 1980s, biochemical studies elucidated the chemical reactions catalysed by these enzymes[76,77]. E1 activates ubiquitin as a thioester of the active site cysteine residue at expenses of ATP[78]. The activated ubiquitin molecule is then transferred to an E2 enzyme via the formation of a new thioester with the catalytic cysteine residue of the E2 enzyme. E2 transfers the

activated ubiquitin moieties to the protein substrate that is specifically bound to a unique ubiquitin ligase: E3. Finally, an isopeptide bond is formed between the C-terminal glycine residue of the ubiquitin (Gly76) and the e-NH2 group of an internal lysine (Lys119) of the protein substrate. Several protein families have been discovered that are evolutionarily related to ubiquitin, as they share the characteristics of the ubiquitin-fold proteins and the capacity to become conjugated to substrates through the action of related E1, E2, and E3 enzymes[79].

Target proteins can be modified by mono- or poly-ubiquitination. The seven lysines in ubiquitin contribute to the assembly of various poly-ubiquitin chains that may be involved in different cellular processes[80]. The K48-linked (Lys 48) poly-ubiquitin chain targets proteins for proteasomal degradation, while the K11-linked poly-ubiquitin chain is involved in endoplasmic reticulum-associated degradation[81] and the K63-linked poly-ubiquitin chain can act in non-proteolytic events, including protein trafficking, DNA repair, and inflammation[82]. Furthermore, ubiquitination can be reversed by the removal of ubiquitin from target proteins by de-ubiquitinating enzymes (DUBs).

Degradation of damaged proteins

The proteasome is a multicatalytic protease involved in the degradation of intracellular proteins. The processing of damaged proteins is mainly mediated by the 20S and 26S proteasomes[76,77].

The 20S proteasome is a large complex, formed of four stacked rings: two alpha and two beta rings, each containing seven different subunits (from $\alpha 1$ to $\alpha 7$ or from $\beta 1$ to $\beta 7$). Three of the beta subunits are responsible for the proteolytic activity. $\beta 1$ exhibits caspase-like activity, while $\beta 2$ exhibits trypsin-like activity, and $\beta 5$, chymotrypsin-like activity. Whereas the beta rings are responsible for proteolytic degradation, the alpha rings perform substrate recognition and regulate substrate access to the proteolytic active centre.

The 26S proteasome is formed by the addition of one or two regulatory 19S subunits to an alpha ring in the 20S core proteasome in an ATP-dependent manner. The binding to this regulator increases the proteasomal activity and allows the proteasome to degrade the substrate proteins that have been tagged through the binding of a chain of ubiquitin, as explained above. The ubiquitin chain acts as a labelled sequence to direct proteins to the 26S proteasome where they are degraded. The 19S subunits first remove the poly-ubiquitin "tags" of the targeted proteins and unfold those proteins. Then, the unfolded proteins are degraded by the 20S core of the 26S proteasome. In this way, while the 20S core proteasome can degrade unfolded proteins in an ATP-independent manner, the 26S proteasome is only capable of degrading natively folded and functional proteins in an ATP- and ubiquitin-

dependent manner[83].

ACTIVATION OF THE PROTEASOME UBIQUITIN SYSTEM (UPS) AND PROTEOLYSIS IN IRI

It has been well established that proteolysis is an important regulatory mechanism that helps maintain homeostasis[77]. The proteasome and the UPS are necessary for the control of protein concentrations, to prevent abnormal accumulations and to modulate regulatory proteins involved in cell metabolism. However, proteolysis can have detrimental effects on organs and tissues after transplantation[84]. Free amino acids in the effluent from human livers was used as a marker to predict postoperative graft function. In fact, the prevention of liver graft proteolysis and proteasome activation can be modulated by the use of additives to organ preservation solutions[85,86]. In this way, it has been demonstrated that lactobionate, a component of UW solution, prevents the release of metalloproteinases during cold preservation[85]. Moreover, the UPS is an energy-dependent system and ATP levels affects 26S proteasome assembly, stability, and function[87]. The following processes are dependent on ATP: The first step in ubiquitin conjugation by E1, which is required for the poly-ubiquitination of proteins; the assembly of alpha and beta rings and ATP-dependent proteases; and the degradation of poly-ubiquinated proteins by the 26S proteasome[79,87]. In addition, it is well known that oxygen deprivation leads to a significant decrease in ATP in liver grafts[34], which could affect liver outcomes.

Therefore, during cold storage of organs, and because of the ATP decrease, the formation of 26S proteasomes and poly-ubiquitin-dependent protein degradation are expected to be impaired. However, it has been well established that a subset of 26S proteasomes appears to be activated as ATP levels decline[70]. In that study, Geng *et al*[70] demonstrated that the activation of the 26S proteasome is a pathophysiologically relevant mechanism in cold ischemic myocardial injury. Such observations imply that a subset of 26S is acting as a harmful protease that is activated when the cellular energy supply decreases. The finding that ATP negatively regulates proteasome activity is consistent with previous results concerning increased proteolysis during cold preservation of human liver grafts[84]. Moreover, hypothermia also affects proteasome activity in isolated perfused rat liver, increasing chymotrypsin-like activity[36].

Degradation of protein substrates by 26S proteasomes requires the 19S regulatory cap to recognize ubiquitin protein conjugates and to regulate the entry of the substrate into the proteolytic cavity of the 20S core. Covalent regulation via phosphorylation of a subunit in the 19S regulatory cap of the proteasome allows for a fast increase in the 26S proteasome activity, which becomes an important regulatory mechanism for

proteasome control[88-90]. In fact, we have previously reported that phosphorylation of the 19S subunit Rpt6 increases in cold perfused rat livers[36].

The combination of proteasome and oxidative stress in IRI

ROS are capable of oxidatively modifying cell structures. Due to the abundance of proteins, the presence of oxidatively modified proteins and aggregates of oxidized proteins has been reported in the cytosol. There is a certain degree of consensus that proteasomal activity degrades oxidized proteins, although the attribution to 20S or 26S is yet to be elucidated. The 26S proteasome was found to be less active in the degradation of oxidatively damaged proteins[91]. A growing body of evidence suggests that the degradation of oxidatively damaged proteins does not require ATP and polyubiquitination of the substrate[92]. Alternatively, oxidative proteins are removed independently of ubiquitin by the ATP-independent 20S proteasome[93].

The proteasome is responsible for the selective degradation of oxidatively damaged proteins. In this sense it has been shown that certain oxidized proteins degrade faster than their native counterparts[94], and, furthermore, it has been shown that inhibition of the proteasome stabilizes the oxidized proteins[95]. During oxidative stress, the ratio of oxidized to reduced glutathione increases[36]. and this redox status of the cells can also modulate protein ubiquitination by reversible S-thiolation[96,67]. This is concurrent with a decrease in the ubiquitin-activating enzyme, E-1 and ubiquitin conjugates. According to some models, glutathiolation of the E1 or E2 components of a ubiquitinated protein protects it from unnecessary degradation. Thus, S-glutathiolation can be regarded as a general mechanism of a redox signal controlling gene expression. In addition, direct oxidative modifications of the proteasome may also occur, including carbonylation, glycoxidation and modification with lipid peroxidation products. Although it is not clear to what extent these modifications affect the proteasome, they could modulate proteasomal activity. It should be noted that an inefficient proteasomal system would result in an accumulation of protein aggregates in the cytoplasm, as has been reported in brain due to aging or Alzheimer's disease[97]; while excessive protein depletion activity may have deleterious effects by affecting detoxifying systems, membranes, or RNA stability[69,84].

PROTEASOME INHIBITION IN LIVER IRI

The use of proteasome inhibitors has been shown to offer protection and maintain the physiological ubiquitin-protein conjugate pool during cold organ preservation[98]. Table 1 summarizes the protective effects of different proteasome inhibitors against IRI during organ preservation. The potential pharmacological role of proteasome inhibitors was first reported by Campbell *et al*[99]. Those

Inhibitor	Organ and condition	Manifestations	Ref.
PS-519	Heart after IRI (rat)	Improved cardiac contractility Improved coronary flow Reduced PMN infiltration	Campbell *et al*[99]
Epoxomicin	Heart after cold ischemia (rat)	Reduced edema formation Preserved ultrastructural integrity	Geng *et al*[70]
MG132	Liver, warm IRI (rat)	Decreased LDH and ALT Increased protein oxidation Decreased antioxidant activities	Alexandrova *et al*[4]
MG132	Liver, cold IRI (rat)	Decreased AST and ALT Reduced inflammation (IL1β and TNFα)	Zaouali *et al*[71]
Bortezomid	Liver, cold IRI (rat)	Decreased AST, ALT and mitochondrial damage Increased bile production Decreased lipid peroxidation Decreased apoptosis (Cyt C and Caspase 3)	Zaouali *et al*[71] Bejaoui *et al*[72]

Table 1 Effect of different proteasome inhibitors on organ preservation

IRI: Ischemia-reperfusion injury.

authors demonstrated that proteasome inhibition can prevent loss of cardiac contractile function in isolated perfused rat heart. The adherence of polymorphonuclear leukocytes to the endothelium was also reduced. However, controversy regarding whether inhibiting the proteasome is beneficial or detrimental to cardiac function continues[95]. Few studies have examined the role of UPS inhibitors as a strategy to reduce damage in liver IRI. In rat liver subjected to warm IRI, the administration of the proteasome inhibitor MG132 decreased LDH and AST levels during ischemia and reperfusion[4]. In addition, the same authors studied whether MG132 can modulate the prooxidant and antioxidant status of rat liver. The results showed that MG132 did not significantly affect liver lipid peroxidation. However, MG132 increased protein carbonyls and decreased the main antioxidant enzyme activities (catalase and superoxide dismutase).

In contrast to that modulation of oxidative stress, the proteasome inhibitor Bortezomib, used at a non-toxic low dose, up-regulates liver antioxidant enzymes in chronic ethanol-fed rats[86]. Exposure to the proteasome inhibitor increased antioxidant defences by enhancing the levels of mRNA and protein expression transcripts of glutathione reductase, glutathione synthetase and glutathione peroxidase, as well as superoxide dismutase in rat liver. The increase in antioxidant defences was concomitant with enhanced 26S proteasome activity. As mentioned by those authors, the beneficial effects of the proteasome inhibitor Bortezomib could be due to the low dose used and to the reversibility of the drug.

In accordance with the previous research, we have recently demonstrated that the addition of the reversible UPS inhibitors Bortezomib and MG132 to UW solution improved steatotic and non-steatotic rat liver preservation in the face of cold IRI[71]. Both inhibitors prevented liver injury, decreasing AST and ALT, and prevented the release of the inflammatory cytokines IL-1 beta and TNF-alpha. The protective effect of Bortezomib was superior to that of MG132. Bortezomib increased bile production, decreased vascular resistance in fatty rat liver through

an increase in nitric oxide generation, prevented lipid peroxidation and mitochondrial damage, and increased AMP-activated protein kinase (AMPK) phosphorylation. It is well known that AMPK phosphorylation is a key process in fatty liver graft preservation, as it can reduce inflammation[9,44].

The supplementation of IGL-1 preservation solution with Bortezomib has also been shown to have protective effects[72], reducing steatotic liver injury and decreasing liver apoptosis (cytochrome c and caspase 3 release) in the face of cold IRI. These effects were partially mediated through the activation of the Akt/mTOR signalling pathway, a key regulator in cell growth and proliferation, and through the phosphorylation of AMPK.

Besides its role in preventing inflammation, AMPK activation also keeps the liver in an energy-conserving state during cold storage. AMPK triggers ATP-producing pathways, balancing the metabolic process towards increasing energy homeostasis in the cell. We have recently found a correlation between AMPK activation, ATP levels, lower proteasome activity and decreased damage in fatty liver grafts preserved in different solutions[73]. As AMPK is regulated and degraded by the UPS, the use of proteasome inhibitors in the preservation solution could avoid AMPK depletion and may contribute to maintaining the beneficial effects of proteasome inhibition after IRI.

CONCLUSION

Remarkable progress has been made over the past few decades regarding the role of the UPS in many cellular processes. However, the function and regulation of the proteasome in organ transplantation still remains unclear. We have proposed that UPS inhibitors reduce IRI in liver grafts *via* up-regulation of AMPK phosphorylation and the consequent preservation of the energy state. As some proteasome inhibitors have been approved for the treatment of different diseases, we propose to explore their use in liver. Well-designed

randomized controlled trials will be needed to evaluate the use of proteasome inhibitors in liver transplantation. The supplementation of low and non-toxic doses of proteasome inhibitors offers a new opportunity for the improvement of organ preservation solutions.

ACKNOWLEDGMENTS

REFERENCES

1   **Nagano K**, Gelman S, Bradley EL Jr, Parks D. Hypothermia, hepatic oxygen supply-demand, and ischemia-reperfusion injury in pigs. *Am J Physiol* 1990; **258**: G910-G918 [PMID: 2360637 DOI: 10.1152/ajpgi.1990.258.6.G910]

2   **Iñiguez M**, Dotor J, Feijoo E, Goñi S, Prieto J, Berasain C, Avila MA. Novel pharmacologic strategies to protect the liver from ischemia-reperfusion injury. *Recent Pat Cardiovasc Drug Discov* 2008; **3**: 9-18 [PMID: 18221124]

3   **Duval M**, Plin C, Elimadi A, Vallerand D, Tillement JP, Morin D, Haddad PS. Implication of mitochondrial dysfunction and cell death in cold preservation--warm reperfusion-induced hepatocyte injury. *Can J Physiol Pharmacol* 2006; **84**: 547-554 [PMID: 16902600 DOI: 10.1139/y06-014]

4   **Alexandrova A**, Petrov L, Georgieva A, Kessiova M, Tzvetanova E, Kirkova M, Kukan M. Effect of MG132 on proteasome activity and prooxidant/antioxidant status of rat liver subjected to ischemia/reperfusion injury. *Hepatol Res* 2008; **38**: 393-401 [PMID: 17927769 DOI: 10.1111/j.1872-034X.2007.00270.x]

5   **Gurusamy KS**, Gonzalez HD, Davidson BR. Current protective strategies in liver surgery. *World J Gastroenterol* 2010; **16**: 6098-6103 [PMID: 21182224 DOI: 10.3748/wjg.v16.i48.6098]

6   **Neves DB**, Rusi MB, Diaz LG, Salvalaggio P. Primary graft dysfunction of the liver: definitions, diagnostic criteria and risk factors. *Einstein* (Sao Paulo) 2016; **14**: 567-572 [PMID: 27783749 DOI: 10.1590/s1679-45082016rw3585]

7   **Hori T**, Uemoto S, Walden LB, Chen F, Baine AM, Hata T, Nguyen JH. Pretreatment of Small-for-Size Grafts In Vivo by γ-Aminobutyric Acid Receptor Regulation against Oxidative Stress-Induced Injury in Rat Split Orthotopic Liver Transplantation. *Int J Hepatol* 2013; **2013**: 149123 [PMID: 24223309 DOI: 10.1155/2013/149123]

8   **Verhoeven CJ**, Farid WR, de Jonge J, Metselaar HJ, Kazemier G, van der Laan LJ. Biomarkers to assess graft quality during conventional and machine preservation in liver transplantation. *J Hepatol* 2014; **61**: 672-684 [PMID: 24798616 DOI: 10.1016/j.jhep.2014.04.031]

9   **Serafín A**, Roselló-Catafau J, Prats N, Gelpí E, Rodés J, Peralta C. Ischemic preconditioning affects interleukin release in fatty livers of rats undergoing ischemia/reperfusion. *Hepatology* 2004; **39**: 688-698 [PMID: 14999687 DOI: 10.1002/hep.20089]

10   **Bejaoui M**, Pantazi E, Calvo M, Folch-Puy E, Serafín A, Pasut G, Panisello A, Adam R, Roselló-Catafau J. Polyethylene Glycol Preconditioning: An Effective Strategy to Prevent Liver Ischemia Reperfusion Injury. *Oxid Med Cell Longev* 2016; **2016**: 9096549 [PMID: 26981166 DOI: 10.1155/2016/9096549]

11   **Peralta C**, Closa D, Hotter G, Gelpí E, Prats N, Roselló-Catafau J. Liver ischemic preconditioning is mediated by the inhibitory action of nitric oxide on endothelin. *Biochem Biophys Res Commun* 1996; **229**: 264-270 [PMID: 8954116]

12   **Eipel C**, Glanemann M, Nuessler AK, Menger MD, Neuhaus P, Vollmar B. Ischemic preconditioning impairs liver regeneration in extended reduced-size livers. *Ann Surg* 2005; **241**: 477-484 [PMID: 15729071]

13   **Li JY**, Gu X, Yin HZ, Zhou Y, Zhang WH, Qin YM. Protective effect of ischemic preconditioning on hepatic ischemia-reperfusion injury by advancing the expressive phase of survivin in rats. *Hepatobiliary Pancreat Dis Int* 2008; **7**: 615-620 [PMID: 19073407]

14   **Koti RS**, Seifalian AM, Davidson BR. Protection of the liver by ischemic preconditioning: a review of mechanisms and clinical applications. *Dig Surg* 2003; **20**: 383-396 [PMID: 12840597]

15   **Amador A**, Grande L, Martí J, Deulofeu R, Miquel R, Solá A, Rodriguez-Laiz G, Ferrer J, Fondevila C, Charco R, Fuster J, Hotter G, García-Valdecasas JC. Ischemic pre-conditioning in deceased donor liver transplantation: a prospective randomized clinical trial. *Am J Transplant* 2007; **7**: 2180-2189 [PMID: 17697262 DOI: 10.1111/j.1600-6143.2007.01914.x]

16   **Doeppner TR**, Doehring M, Kaltwasser B, Majid A, Lin F, Bähr M, Kilic E, Hermann DM. Ischemic Post-Conditioning Induces Post-Stroke Neuroprotection via Hsp70-Mediated Proteasome Inhibition and Facilitates Neural Progenitor Cell Transplantation. *Mol Neurobiol* 2017; **54**: 6061-6073 [PMID: 27699598 DOI: 10.1007/s12035-016-0137-3]

17   **Belzer FO**, Southard JH. Principles of solid-organ preservation by cold storage. *Transplantation* 1988; **45**: 673-676 [PMID: 3282347]

18   **Ferrigno A**, Rizzo V, Boncompagni E, Bianchi A, Gringeri E, Neri D, Richelmi P, Freitas I, Cillo U, Vairetti M. Machine perfusion at 20°C reduces preservation damage to livers from non-heart beating donors. *Cryobiology* 2011; **62**: 152-158 [PMID: 21315707 DOI: 10.1016/j.cryobiol.2011.02.004]

19   **Schlegel A**, Dutkowski P. Role of hypothermic machine perfusion in liver transplantation. *Transpl Int* 2015; **28**: 677-689 [PMID: 24852621 DOI: 10.1111/tri.12354]

20   **Hessheimer AJ**, Fondevila C, García-Valdecasas JC. Extracorporeal machine liver perfusion: are we warming up? *Curr Opin Organ Transplant* 2012; **17**: 143-147 [PMID: 22273595 DOI: 10.1097/MOT.0b013e328351082a]

21   **Sola A**, Roselló-Catafau J, Gelpí E, Hotter G. Fructose-1,6-biphosphate in rat intestinal preconditioning: involvement of nitric oxide. *Gut* 2001; **48**: 168-175 [PMID: 11156636]

22   **Padrissa-Altés S**, Franco-Gou R, Boillot O, Serafín A, Rimola A, Arroyo V, Rodés J, Peralta C, Roselló-Catafau J. Effect of angiotensin II and bradykinin inhibition in rat reduced-size liver transplantation. *Liver Transpl* 2009; **15**: 313-320 [PMID: 19242996 DOI: 10.1002/lt.21693]

23   **Zaouali MA**, Ben Abdennebi H, Padrissa-Altés S, Mahfoudh-Boussaid A, Roselló-Catafau J. Pharmacological strategies against cold ischemia reperfusion injury. *Expert Opin Pharmacother* 2010; **11**: 537-555 [PMID: 20163266 DOI: 10.1517/14656560903547836]

24   **Bejaoui M**, Pantazi E, Folch-Puy E, Baptista PM, García-Gil A, Adam R, Roselló-Catafau J. Emerging concepts in liver graft preservation. *World J Gastroenterol* 2015; **21**: 396-407 [PMID: 25593455 DOI: 10.3748/wjg.v21.i2.396]

25   **Carbonell T,** Alva N, Palomeque J. Mechanisms activated by induced hypothermia: positive and adverse effects. In: Delgado JIV, Garza VGF. Hypothermia: Prevention, Recognition and Treatment. New York: Nova Publishers; **2012**: 109-122

26   **Halliwell B**, Gutteridge JM. Free radicals and antioxidant protection: mechanisms and significance in toxicology and disease. *Hum Toxicol* 1988; **7**: 7-13 [PMID: 3278973]

27   **Gutteridge JM**, Halliwell B. Reoxygenation injury and antioxidant protection: a tale of two paradoxes. *Arch Biochem Biophys* 1990; **283**: 223-226 [PMID: 2177320]

28   **Granger DN**, Kvietys PR. Reperfusion injury and reactive oxygen species: The evolution of a concept. *Redox Biol* 2015; **6**: 524-551 [PMID: 26484802 DOI: 10.1016/j.redox.2015.08.020]

29   **Jung T**, Catalgol B, Grune T. The proteasomal system. *Mol Aspects Med* 2009; **30**: 191-296 [PMID: 19371762 DOI: 10.1016/j.mam.2009.04.001]

30   **Ciechanover A**. Intracellular protein degradation: from a vague idea thru the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting. *Biochim Biophys Acta* 2012; **1824**: 3-13 [PMID: 21435401 DOI: 10.1016/

j.bbapap.2011.03.007]

31   **Shang F**, Taylor A. Ubiquitin-proteasome pathway and cellular responses to oxidative stress. *Free Radic Biol Med* 2011; **51**: 5-16 [PMID: 21530648 DOI: 10.1016/j.freeradbiomed.2011.03.031]

32   **Guibert EE**, Petrenko AY, Balaban CL, Somov AY, Rodriguez JV, Fuller BJ. Organ Preservation: Current Concepts and New Strategies for the Next Decade. *Transfus Med Hemother* 2011; **38**: 125-142 [PMID: 21566713 DOI: 10.1159/000327033]

33   **Olschewski P**, Gass P, Ariyakhagorn V, Jasse K, Hunold G, Menzel M, Schöning W, Schmitz V, Neuhaus P, Puhl G. The influence of storage temperature during machine perfusion on preservation quality of marginal donor livers. *Cryobiology* 2010; **60**: 337-343 [PMID: 20233587 DOI: 10.1016/j.cryobiol.2010.03.005]

34   **Vajdová K**, Graf R, Clavien PA. ATP-supplies in the cold-preserved liver: A long-neglected factor of organ viability. *Hepatology* 2002; **36**: 1543-1552 [PMID: 12447882 DOI: 10.1053/jhep.2002.37189]

35   **Stefanovich P**, Ezzell RM, Sheehan SJ, Tompkins RG, Yarmush ML, Toner M. Effects of hypothermia on the function, membrane integrity, and cytoskeletal structure of hepatocytes. *Cryobiology* 1995; **32**: 389-403 [PMID: 7656572]

36   **Carbonell T**, Alva N, Sanchez-Nuño S, Dewey S, Gomes AV. Subnormothermic Perfusion in the Isolated Rat Liver Preserves the Antioxidant Glutathione and Enhances the Function of the Ubiquitin Proteasome System. *Oxid Med Cell Longev* 2016; **2016**: 9324692 [PMID: 27800122 DOI: 10.1155/2016/9324692]

37   **Bruinsma BG**, Yeh H, Ozer S, Martins PN, Farmer A, Wu W, Saeidi N, Op den Dries S, Berendsen TA, Smith RN, Markmann JF, Porte RJ, Yarmush ML, Uygun K, Izamis ML. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. *Am J Transplant* 2014; **14**: 1400-1409 [PMID: 24758155 DOI: 10.1111/ajt.12727]

38   **Op den Dries S**, Sutton ME, Karimian N, de Boer MT, Wiersema-Buist J, Gouw AS, Leuvenink HG, Lisman T, Porte RJ. Hypothermic oxygenated machine perfusion prevents arteriolonecrosis of the peribiliary plexus in pig livers donated after circulatory death. *PLoS One* 2014; **9**: e88521 [PMID: 24551114 DOI: 10.1371/journal.pone.0088521]

39   **Ferrigno A**, Di Pasqua LG, Berardo C, Siciliano V, Rizzo V, Mannucci B, Richelmi P, Croce AC, Vairetti M. Liver Graft Susceptibility during Static Cold Storage and Dynamic Machine Perfusion: DCD versus Fatty Livers. *Int J Mol Sci* 2017; **19**: [PMID: 29301219 DOI: 10.3390/ijms19010109]

40   **Vairetti M**, Ferrigno A, Carlucci F, Tabucchi A, Rizzo V, Boncompagni E, Neri D, Gringeri E, Freitas I, Cillo U. Subnormothermic machine perfusion protects steatotic livers against preservation injury: a potential for donor pool increase? *Liver Transpl* 2009; **15**: 20-29 [PMID: 19109848 DOI: 10.1002/lt.21581]

41   **Brockmann J**, Reddy S, Coussios C, Pigott D, Guirriero D, Hughes D, Morovat A, Roy D, Winter L, Friend PJ. Normothermic perfusion: a new paradigm for organ preservation. *Ann Surg* 2009; **250**: 1-6 [PMID: 19561463 DOI: 10.1097/SLA.0b013e3181a63c10]

42   **Ravikumar R**, Leuvenink H, Friend PJ. Normothermic liver preservation: a new paradigm? *Transpl Int* 2015; **28**: 690-699 [PMID: 25847684 DOI: 10.1111/tri.12576]

43   **Hoyer DP**, Paul A, Luer S, Reis H, Efferz P, Minor T. End-ischemic reconditioning of liver allografts: Controlling the rewarming. *Liver Transpl* 2016; **22**: 1223-1230 [PMID: 27398813 DOI: 10.1002/lt.24515]

44   **Vairetti M**, Ferrigno A, Rizzo V, Richelmi P, Boncompagni E, Neri D, Freitas I, Cillo U. Subnormothermic machine perfusion protects against rat liver preservation injury: a comparative evaluation with conventional cold storage. *Transplant Proc* 2007; **39**: 1765-1767 [PMID: 17692606 DOI: 10.1016/j.transproceed.2007.06.002]

45   **op den Dries S**, Karimian N, Sutton ME, Westerkamp AC, Nijsten MW, Gouw AS, Wiersema-Buist J, Lisman T, Leuvenink HG, Porte RJ. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. *Am J Transplant* 2013; **13**: 1327-1335 [PMID: 23463950 DOI: 10.1111/ajt.12187]

46   **Duret C**, Moreno D, Balasiddaiah A, Roux S, Briolotti P, Raulet E, Herrero A, Ramet H, Biron-Andreani C, Gerbal-Chaloin S, Ramos J, Navarro F, Hardwigsen J, Maurel P, Aldabe R, Daujat-Chavanieu M. Cold Preservation of Human Adult Hepatocytes for Liver Cell Therapy. *Cell Transplant* 2015; **24**: 2541-2555 [PMID: 25622096 DOI: 10.3727/096368915X687020]

47   **Ben Abdennebi H**, Steghens JP, Margonari J, Ramella-Virieux S, Barbieux A, Boillot O. High-Na+ low-K+ UW cold storage solution reduces reperfusion injuries of the rat liver graft. *Transpl Int* 1998; **11**: 223-230 [PMID: 9638853]

48   **Boudjema K**, Grandadam S, Compagnon P, Salamé E, Wolf P, Ducerf C, Le Treut P, Soubrane O, Cherqui D, Mouchel C, Renault A, Bellissant E. Efficacy and safety of Celsior preservation fluid in liver transplantation: one-year follow up of a prospective, multicenter, non-randomized study. *Clin Transplant* 2012; **26**: 199-207 [PMID: 21517997 DOI: 10.1111/j.1399-0012.2011.01447.x]

49   **Stewart ZA**, Cameron AM, Singer AL, Montgomery RA, Segev DL. Histidine-Tryptophan-Ketoglutarate (HTK) is associated with reduced graft survival in deceased donor livers, especially those donated after cardiac death. *Am J Transplant* 2009; **9**: 286-293 [PMID: 19067658 DOI: 10.1111/j.1600-6143.2008.02478.x]

50   **Zaouali MA**, Ben Abdennebi H, Padrissa-Altés S, Alfany-Fernandez I, Rimola A, Roselló-Catafau J. How Institut Georges Lopez preservation solution protects nonsteatotic and steatotic livers against ischemia-reperfusion injury. *Transplant Proc* 2011; **43**: 77-79 [PMID: 21335159 DOI: 10.1016/j.transproceed.2010.12.026]

51   **Halliwell B**, Gutteridge JMC. Free radicals in biology and medicin. Oxford: Oxford University Press; 1999. ISBN: 0198500459

52   **Bessems M**, 't Hart NA, Tolba R, Doorschodt BM, Leuvenink HG, Ploeg RJ, Minor T, van Gulik TM. The isolated perfused rat liver: standardization of a time-honoured model. *Lab Anim* 2006; **40**: 236-246 [PMID: 16803641 DOI: 10.1258/002367706777611460]

53   **Kron P**, Schlegel A, Mancina L, Clavien PA, Dutkowski P. Hypothermic oxygenated perfusion (HOPE) for fatty liver grafts in rats and humans. *J Hepatol* 2017; Epub ahead of print [PMID: 28870676 DOI: 10.1016/j.jhep.2017.08.028]

54   **Polat KY**, Aydinli B, Polat O, Aydin U, Yazici P, Ozturk G, Gundogdu C, Kiziltunc A. The protective effect of aprotinin and alpha-tocopherol on ischemia-reperfusion injury of the rat liver. *Transplant Proc* 2008; **40**: 63-68 [PMID: 18261548 DOI: 10.1016/j.transproceed.2007.11.047]

55   **Gulec B**, Coskun K, Yigitler C, Yigit T, Aydin A, Oner K. Ischemia-reperfusion injury in the liver during renal transplantation: does perfusion solution play any role? *Transplant Proc* 2008; **40**: 59-62 [PMID: 18261547 DOI: 10.1016/j.transproceed.2007.11.023]

56   **Qi Q**, Bie P. Different roles of hepatic hypothermic ischemia and ischemic preconditioning in chemically induced hepatocarcinogenesis in rats. *J Surg Res* 2014; **189**: 213-221 [PMID: 24725680 DOI: 10.1016/j.jss.2014.03.008]

57   **Thompson-Gorman SL**, Zweier JL. Evaluation of the role of xanthine oxidase in myocardial reperfusion injury. *J Biol Chem* 1990; **265**: 6656-6663 [PMID: 2157706]

58   **Peralta C**, Closa D, Xaus C, Gelpí E, Roselló-Catafau J, Hotter G. Hepatic preconditioning in rats is defined by a balance of adenosine and xanthine. *Hepatology* 1998; **28**: 768-773 [PMID: 9731571 DOI: 10.1002/hep.510280325]

59   **Martí R**, Múrio E, Varela E, Bilbao I, Pascual C, Margarit C, Segura RM. Xanthine oxidoreductase and preservation injury in human liver transplantation. *Transplantation* 2004; **77**: 1239-1245 [PMID: 15114092 DOI: 10.1097/01.TP.0000120384.52033.BC]

60   **Jaeschke H**. Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G15-G26 [PMID: 12488232 DOI: 10.1152/ajpgi.00342.2002]

61   **Lee WY**, Koh EJ, Lee SM. A combination of ischemic preconditioning and allopurinol protects against ischemic injury through a nitric oxide-dependent mechanism. *Nitric Oxide* 2012; **26**: 1-8 [PMID: 22119149 DOI: 10.1016/j.niox.2011.11.002]

62   **Marden JJ**, Zhang Y, Oakley FD, Zhou W, Luo M, Jia HP, McCray PB Jr, Yaniv M, Weitzman JB, Engelhardt JF. JunD

protects the liver from ischemia/reperfusion injury by dampening AP-1 transcriptional activation. *J Biol Chem* 2008; **283**: 6687-6695 [PMID: 18182393 DOI: 10.1074/jbc.M705606200]

63   **Shi S**, Xue F. Current Antioxidant Treatments in Organ Transplantation. *Oxid Med Cell Longev* 2016; **2016**: 8678510 [PMID: 27403232 DOI: 10.1155/2016/8678510]

64   **Junnarkar SP**, Tapuria N, Dutt N, Fuller B, Seifalian AM, Davidson BR. Bucillamine improves hepatic microcirculation and reduces hepatocellular injury after liver warm ischaemia-reperfusion injury. *HPB* (Oxford) 2009; **11**: 264-273 [PMID: 19590658 DOI: 10.1111/J.1477-2574.2009.00054.X]

65   **de Oca J**, Cuadrado S, Vallet J, Benasco C, Martín E, Ardanuy C, Closa D, Hotter G, Jaurrieta E. Protective effects of lazaroid U74389G on intestinal graft after heterotopic small bowel transplantation in rats. *J Surg Res* 1998; **75**: 18-23 [PMID: 9614851 DOI: 10.1006/jsre.1997.5244]

66   **Yilmaz HR**, Uz E, Yucel N, Altuntas I, Ozcelik N. Protective effect of caffeic acid phenethyl ester (CAPE) on lipid peroxidation and antioxidant enzymes in diabetic rat liver. *J Biochem Mol Toxicol* 2004; **18**: 234-238 [PMID: 15452882 DOI: 10.1002/jbt.20028]

67   **Demasi M**, Netto LE, Silva GM, Hand A, de Oliveira CL, Bicev RN, Gozzo F, Barros MH, Leme JM, Ohara E. Redox regulation of the proteasome via S-glutathionylation. *Redox Biol* 2013; **2**: 44-51 [PMID: 24396728 DOI: 10.1016/J.REDOX.2013.12.003]

68   **Zhou H**, Zhu J, Yue S, Lu L, Busuttil RW, Kupiec-Weglinski JW, Wang X, Zhai Y. The Dichotomy of Endoplasmic Reticulum Stress Response in Liver Ischemia-Reperfusion Injury. *Transplantation* 2016; **100**: 365-372 [PMID: 26683513 DOI: 10.1097/TP.0000000000001032]

69   **Baker TA**, Geng Q, Romero J, Picken MM, Gamelli RL, Majetschak M. Prolongation of myocardial viability by proteasome inhibition during hypothermic organ preservation. *Biochem Biophys Res Commun* 2010; **401**: 548-553 [PMID: 20875792]

70   **Geng Q**, Romero J, Saini V, Baker TA, Picken MM, Gamelli RL, Majetschak M. A subset of 26S proteasomes is activated at critically low ATP concentrations and contributes to myocardial injury during cold ischemia. *Biochem Biophys Res Commun* 2009; **390**: 1136-1141 [PMID: 19944202 DOI: 10.1016/j.bbrc.2009.10.067]

71   **Zaouali MA**, Bardag-Gorce F, Carbonell T, Oliva J, Pantazi E, Bejaoui M, Ben Abdennebi H, Rimola A, Rosselló-Catafau J. Proteasome inhibitors protect the steatotic and non-steatotic liver graft against cold ischemia reperfusion injury. *Exp Mol Pathol* 2013; **94**: 352-359 [PMID: 23305864]

72   **Bejaoui M**, Zaouali MA, Folch-Puy E, Pantazi E, Bardag-Gorce F, Carbonell T, Oliva J, Rimola A, Abdennebi HB, Rosselló-Catafau J. Bortezomib enhances fatty liver preservation in Institut George Lopez-1 solution through adenosine monophosphate activated protein kinase and Akt/mTOR pathways. *J Pharm Pharmacol* 2014; **66**: 62-72 [PMID: 24127984 DOI: 10.1111/jphp.12154]

73   **Panisello-Rosselló A**, Verde E, Amine Zaouali M, Flores M, Alva N, Lopez A, Folch-Puy E, Carbonell T, Hotter G, Adam R, Rosselló-Catafau J. The Relevance of the UPS in Fatty Liver Graft Preservation: A New Approach for IGL-1 and HTK Solutions. *Int J Mol Sci* 2017; **18**: [PMID: 29088097]

74   **Goldstein G**, Scheid M, Hammerling U, Schlesinger DH, Niall HD, Boyse EA. Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. *Proc Natl Acad Sci USA* 1975; **72**: 11-15 [PMID: 1078892 DOI: 10.1073/pnas.72.1.11]

75   **Ciechanover A**, Elias S, Heller H, Hershko A. "Covalent affinity" purification of ubiquitin-activating enzyme. *J Biol Chem* 1982; **257**: 2537-2542 [PMID: 6277904]

76   **Hershko A**, Ciechanover A. The ubiquitin system. *Annu Rev Biochem* 1998; **67**: 425-479 [PMID: 9759494 DOI: 10.1007/978-1-4899-1922-9_1]

77   **Ciechanover A**. Intracellular protein degradation: from a vague idea through the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting. *Bioorg Med Chem* 2013; **21**: 3400-3410 [PMID: 23485445 DOI: 10.1016/j.bmc.2013.01.056]

78   **Hershko A**, Heller H, Elias S, Ciechanover A. Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. *J Biol Chem* 1983; **258**: 8206-8214 [PMID: 6305978]

79   **Hameed DS**, Sapmaz A, Ovaa H. How Chemical Synthesis of Ubiquitin Conjugates Helps To Understand Ubiquitin Signal Transduction. *Bioconjug Chem* 2017; **28**: 805-815 [PMID: 27077728]

80   **Kim W**, Bennett EJ, Huttlin EL, Guo A, Li J, Possemato A, Sowa ME, Rad R, Rush J, Comb MJ, Harper JW, Gygi SP. Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol Cell* 2011; **44**: 325-340 [PMID: 21906983 DOI: 10.1016/j.molcel.2011.08.025]

81   **Xu P**, Duong DM, Seyfried NT, Cheng D, Xie Y, Robert J, Rush J, Hochstrasser M, Finley D, Peng J. Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. *Cell* 2009; **137**: 133-145 [PMID: 19345192]

82   **Chen ZJ**, Sun LJ. Nonproteolytic functions of ubiquitin in cell signaling. *Mol Cell* 2009; **33**: 275-286 [PMID: 19217402 DOI: 10.1016/j.molcel.2009.01.014]

83   **Jung T**, Grune T. The proteasome and the degradation of oxidized proteins: Part I-structure of proteasomes. *Redox Biol* 2013; **1**: 178-182 [PMID: 24024151 DOI: 10.1016/j.redox.2013.01.004]

84   **Calmus Y**, Cynober L, Dousset B, Lim SK, Soubrane O, Conti F, Houssin D, Giboudeau J. Evidence for the detrimental role of proteolysis during liver preservation in humans. *Gastroenterology* 1995; **108**: 1510-1516 [PMID: 7729644 DOI: 10.1016/0016-5085(95)90701-7]

85   **Adam R**, Delvart V, Karam V, Ducerf C, Navarro F, Letoublon C, Belghiti J, Pezet D, Castaing D, Le Treut YP, Gugenheim J, Bachellier P, Pirenne J, Muiesan P; ELTR contributing centres, the European Liver, Intestine Transplant Association (ELITA). Compared efficacy of preservation solutions in liver transplantation: a long-term graft outcome study from the European Liver Transplant Registry. *Am J Transplant* 2015; **15**: 395-406 [PMID: 25612492 DOI: 10.1111/ajt.13060]

86   **Zaouali MA**, Panisello-Rosselló A, Lopez A, Castro Benítez C, Folch-Puy E, García-Gil A, Carbonell T, Adam R, Rosselló-Catafau J. Relevance of proteolysis and proteasome activation in fatty liver graft preservation: An Institut Georges Lopez-1 vs University of Wisconsin appraisal. *World J Gastroenterol* 2017; **23**: 4211-4221 [PMID: 28694661 DOI: 10.3748/wjg.v23.i23.4211]

87   **Majetschak M**. Regulation of the proteasome by ATP: implications for ischemic myocardial injury and donor heart preservation. *Am J Physiol Heart Circ Physiol* 2013; **305**: H267-H278 [PMID: 23709597 DOI: 10.1152/ajpheart.00206.2012]

88   **Zhang F**, Hu Y, Huang P, Toleman CA, Paterson AJ, Kudlow JE. Proteasome function is regulated by cyclic AMP-dependent protein kinase through phosphorylation of Rpt6. *J Biol Chem* 2007; **282**: 22460-22471 [PMID: 17565987]

89   **Djakovic SN**, Marquez-Lona EM, Jakawich SK, Wright R, Chu C, Sutton MA, Patrick GN. Phosphorylation of Rpt6 regulates synaptic strength in hippocampal neurons. *J Neurosci* 2012; **32**: 5126-5131 [PMID: 22496558]

90   **Jarome TJ**, Kwapis JL, Ruenzel WL, Helmstetter FJ. CaMKII, but not protein kinase A, regulates Rpt6 phosphorylation and proteasome activity during the formation of long-term memories. *Front Behav Neurosci* 2013; **7**: 115 [PMID: 24009566]

91   **Grune T**, Reinheckel T, Davies KJ. Degradation of oxidized proteins in K562 human hematopoietic cells by proteasome. *J Biol Chem* 1996; **271**: 15504-15509 [PMID: 8663134]

92   **Höhn TJ**, Grune T. The proteasome and the degradation of oxidized proteins: part III-Redox regulation of the proteasomal system. *Redox Biol* 2014; **2**: 388-394 [PMID: 24563857 DOI: 10.1016/j.redox.2013.12.029]

93   **Davies KJ**. Degradation of oxidized proteins by the 20S proteasome. *Biochimie* 2001; **83**: 301-310 [PMID: 11295490]

94   **Shang F**, Nowell TR Jr, Taylor A. Removal of oxidatively damaged proteins from lens cells by the ubiquitin-proteasome

pathway. *Exp Eye Res* 2001; **73**: 229-238 [PMID: 11446773 DOI: 10.1006/exer.2001.1029]

95   **Gomes AV**, Zong C, Ping P. Protein degradation by the 26S proteasome system in the normal and stressed myocardium. *Antioxid Redox Signal* 2006; **8**: 1677-1691 [PMID: 16987021 DOI: 10.1089/ars.2006.8.1677]

96   **Jahngen-Hodge J**, Obin MS, Gong X, Shang F, Nowell TR Jr, Gong J, Abasi H, Blumberg J, Taylor A. Regulation of ubiquitin-conjugating enzymes by glutathione following oxidative stress. *J Biol Chem* 1997; **272**: 28218-28226 [PMID: 9353272 DOI: 10.1074/jbc.272.45.28218]

97   **Bonet-Costa V**, Pomatto LC, Davies KJ. The Proteasome and Oxidative Stress in Alzheimer's Disease. *Antioxid Redox Signal* 2016; **25**: 886-901 [PMID: 27392670 DOI: 10.1089/ars.2016.6802]

98   **Majetschak M**, Patel MB, Sorell LT, Liotta C, Li S, Pham SM. Cardiac proteasome dysfunction during cold ischemic storage and reperfusion in a murine heart transplantation model. *Biochem Biophys Res Commun* 2008; **365**: 882-888 [PMID: 18053796]

99   **Campbell B**, Adams J, Shin YK, Lefer AM. Cardioprotective effects of a novel proteasome inhibitor following ischemia and reperfusion in the isolated perfused rat heart. *J Mol Cell Cardiol* 1999; **31**: 467-476 [PMID: 10093058]

Basic Study

Stomach wall structure and vessels imaging by acoustic resolution photoacoustic microscopy

Cheng Wang, Yu-Fei Lu, Chun-Miao Cai, Hua-Zhong Xiang, Gang Zheng

Cheng Wang, Yu-Fei Lu, Hua-Zhong Xiang, Gang Zheng, Institute of Biomedical Optics and Optometry, School of Medical Instrument and Food Engineering, University of Shanghai for Science and Technology, Shanghai 200093, China

Chun-Miao Cai, Department of Pathology, Shanghai Tenth People's Hospital Affiliated to Tongji University, Shanghai 200093, China

ORCID number: Cheng Wang (0000-0002-6293-1607); Yu-Fei Lu (0000-0002-1446-0070); Chun-Miao Cai (0000-0003-3692-5120); Hua-Zhong Xiang (0000-0002-2544-4850); Gang Zheng (0000-0002-6818-0509).

Author contributions: Wang C designed and performed the research; Wang C and Lu YF wrote the paper; Cai CM harvested experimental samples and provided the pathology images; Wang C, Lu YF, Xiang HZ, and Gang Z performed the photoacoustic imaging experiment and processed the experiment data.

Institutional review board statement: This study was approved by the Laboratory Animal Ethics Committee of Shanghai Tenth People's Hospital. The design is reasonable and in line with the animal requirement. The ethics committee agreed to carry out a formal experiment.

Conflict-of-interest statement: To the best of our knowledge, no conflict of interest exists.

Data sharing statement: No additional data are available.

Abstract

AIM
To image stomach wall blood vessels and tissue, layer-by-layer.

METHODS
We built up the acoustic resolution photoacoustic microscopy (AR-PAM) system for imaging layered tissues, such as the stomach wall. A tunable dye laser system was coupled to a fiber bundle. The fibers of the bundle were placed in nine directions with an incident angle of 45° around a high-frequency ultrasound transducer attached to the acoustic lens. This structure formed a dark field on the tissue surface under the acoustic lens and the nine light beams from the fibers to be combined near the focal point of the acoustic lens. The sample piece was cut from a part of the porcine stomach into a petri dish. In order to realize photoacoustic depth imaging of tumor, we designed a tumor model based on indocyanine green (ICG) dye. The ICG solution (concentration of 129 μM/mL)

was mixed into molten gel, and then a gel mixture of ICG (concentration of 12.9 μM/mL) was injected into the stomach submucosa. The injection quantity was controlled by 0.1 mL to make a small tumor model.

RESULTS
An acoustic resolution photoacoustic microscopy based on fiber illumination was established and an axial resolution of 25 μm and a lateral resolution of 50 μm in its focal zone range of 500 μm has been accomplished. We tuned the laser wavelength to 600 nm. The photoacoustic probe was driven to do B-scan imaging in tissue thickness of 200 μm. The photoacoustic micro-image of mucosa and submucosa of the tissue have been obtained and compared with a pathological photograph of the tissue stained by hematoxylin-eosin staining. We have observed more detailed internal structure of the tissue. We also utilized this photoacoustic microscopy to image blood vessels inside the submucosa. High contrast imaging of the submucosa tumor model was obtained using ICG dye.

CONCLUSION
This AR-PAM is able to image layer-by-layer construction and some blood vessels under mucosa in the stomach wall without any contrast agents.

Key words: Photoacoustic imaging; Stomach; Layered tissue; Acoustic resolution; Fiber

Core tip: In order to image layered tissue and blood vessels, acoustic resolution photoacoustic microscopy based on fiber illumination was established and an axial resolution of 25 μm and a lateral resolution of 50 μm in its focal zone range of 500 μm was accomplished. Layer-by-layer imaging of the stomach tissue and stomach mucosa blood vessels were obtained. High contrast imaging of the submucosa tumor model was obtained using ICG dye.

INTRODUCTION

Stomach submucosal tumors (SMT) are any intramural growth underneath the mucosa of the gastrointestinal tract[1]. These tumors are very hard to find because they are usually asymptomatic and therefore most often discovered as accidental findings during surgery, autopsy, and so on. When examining submucosal tumors, standard optical stomach endoscopy, capsule optical endoscopy and push-and-pull enteroscopy together with barium contrast X-ray do not alone provide sufficient information. Endoscopic ultrasound (EUS), computed tomography (CT) and magnetic resonance imaging (MRI) are recommended as supplementary tools[2] because CT and MRI often lack either sufficient spatial resolution or satisfactory contrast (or both) to be effective for early-stage tumor imaging[3]. Optimal EUS imaging of an SMT needs submersion of the tumor under water. However, benign SMTs, for example the submucosal inflammation, cannot be distinguished endosonographically[4]. Therefore, early-phase tumor detection or in situ characterization of diseased tissue is challenging for EUS. Because the mechanism of EUS imaging is ultrasound imaging, this is based on tissue bulk mechanical properties. Tumor tissue boundaries and blood vessel structures are clinically relevant and provide necessary information for assessing disease stage and planning treatment therapies.

Recently, optical endoscopic imaging modalities, like narrow band imaging endoscopy[5], endoscopic optical coherent tomography[6] and confocal laser endomicroscopy[7] have been developed. They can detect tissue or tissue changes with high sensitivity and high spatial resolution. Because of the strong optical scattering properties of tissue, these techniques are unable to image targets beyond a 1-2 mm depth. Photoacoustic imaging has a lot of advantages, such as, endogenous optical chromophore contrast enables label-free imaging of the microvasculature with a high resolution[8], and *in vivo* blood vessel photoacoustic imaging can cover the length scale from a superficial capillary[9] to an abdominal aorta[10], demonstrating the potential of photoacoustic imaging to bridge the resolution and penetration gaps between microvascular microscopy, clinical angiography and so on[11,12]. This paper's aim is to demonstrate that photoacoustic imaging is able to image the layered tissue and blood vessels under the surface of the tissue. In the future, we can design and develop an photoacoustic endoscopic imaging system to image deeper tissue with higher resolution for diagnosis and treatment.

MATERIALS AND METHODS

AR-PAM system
The acoustic resolution photoacoustic microscopy (AR-PAM) system for stomach wall imaging was shown in Figure 1A. A tunable dye laser system pumped by a Q-switch Nd:YAG laser (ND6000, continuum) was used to provide laser pulse with a pulse repetition of 10 Hz and a pulse width of 5.5 ns. The tunable range of the laser light was 415 nm to 940 nm. A pair of concave and convex lenses expanded and collimated the light beam to approximately 5 mm in diameter. Then coupling to a fiber bundle that was composed by nine optical fibers (FT400UMT, Thorlabs) which have a 400 μm core
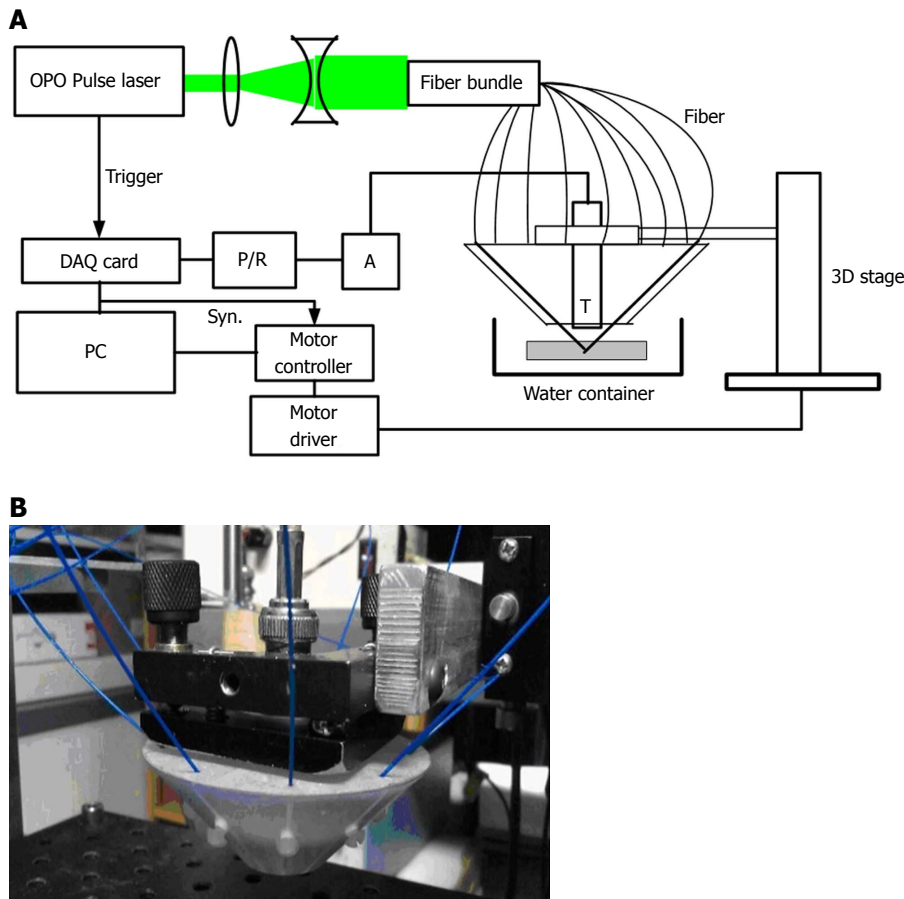
A

B

Figure 1 **Schematic of the acoustic resolution photoacoustic microscopy system (A) and photograph of the photoacoustic probe (B).** P/R: Pulser/receiver; A: Preamplifier; T: Transducer, PC: Personal computer.

diameter and numerical aperture of 0.39 were placed in nine directions (40° intervals in 360°) around a high-frequency (50-MHz, bandwidth of 30 MHz) ultrasound transducer attached with the acoustic lens (the f/# is 1.3 and focal length is 4 mm). The cross angle between the fibers with the transducer axis direction were set to 45°, so that it formed a dark field on the tissue surface under the acoustic lens and the nine light beams from the fibers were combined near the focal point of the acoustic lens, *i.e.* it is then weakly focused into the tissue with the focal region coaxially overlapping the ultrasonic focus inside the tissue. In an optically transparent medium, the optical focus is about 2 mm in diameter, which is much wider than the ultrasonic focus. The focal acoustic transducer and output end of the fibers were fastened with a holder made by a 3D printer. We can refer to it as a photoacoustic probe (Figure 1B). According to the parameters of the ultrasonic transducer, an axial resolution of 25 μm and a lateral resolution of 50 μm in its focal zone range of 500 μm can be accomplished. The photoacoustic probe is translated in a water bath. A window at the bottom of the water container is sealed with an optically and ultrasonically transparent disposable polyethylene membrane (thickness: 0.04 mm). After commercial ultrasound gel is applied to the region of interest on the sample for acoustic coupling, the sample is placed between the sample supporter and

the water container for imaging. The photoacoustic wave is recorded at each location of the ultrasonic transducer and subsequently converted into a one-dimensional (1D) depth-resolved image (A-scan) based on the sound velocity in soft tissue (1.54 mm/us). Images were generated by one dimension (B-Scan) in the X or Y direction or two-dimensional (C-Scan) raster scanning of the photoacoustic probe in the X-Y plane with a step size of 30 μm. In addition to, we can also scan in the Z direction with a step of 200 μm for producing an image of the deeper tissue by utilizing deeply penetrable diffused light to excite photoacoustic signals. At each scanning position, signals were recorded by an 8 bits digitizer card (DP1400, Agilent Tech, United States) after it was amplified by a preamplifier (AU-2A-0150-BNC, MTEQ, United States) and then amplified and filtered (high pass 1 MHz) by a pulser/receiver (5073PR, Olympus, Japan). No signal averaging is performed. As shown in Figure 2, photoacoustic imaging of a hair with a diameter of 80 μm were obtained with this photoacoustic system for verifying the imaging ability. Figure 2A was a B-scan image and Figure 2B was a C-scan image of the hair.

Preparing a porcine stomach wall sample
A porcine stomach was received the day after the animal was sacrificed for an unrelated study. The sample piece was cut from a part of the porcine stomach into a petri

Figure 2 **Photoacoustic image of a hair with a diameter of 80 μm.** A: B-scan imaging. B: C-scan imaging.

Figure 3 **Imaging of layer-by-layer structure of stomach wall.**

dish with a diameter of 140 mm and a thickness of 22 mm. In order to obtain a photoacoustic depth image of the tumor, we designed a tumor model based on indocyanine green (ICG) dye, wherein the ICG solution at a concentration of 129 μM/mL was mixed into molten gel, and then the gel mixture of ICG at a concentration of 12.9 μM/mL was injected into the stomach submucosa at a 2-3 mm position. The injection quantity was controlled to 0.1 mL to make a small tumor model.

RESULTS

In order to verify photoacoustic imaging can also be used to implement stratified imaging of the stomach wall, we tuned the laser wavelength to 600 nm. The photoacoustic probe obtained many B-scan images at tissue thickness direction with a lateral scanning step of 200 μm. Then, all of the B-scan images were combined to complete an image of the layered structure of the stomach wall (Figure 3). A particle with the diameter of 300 μm was placed on the surface of stomach tissue, in order to indicate the surface position of the tissue. As shown in Figure 4, the photoacoustic microimage of the mucosa and submucosa of the tissue have been obtained

and compared with a pathological photograph of the tissue stained by hematoxylin-eosin staining. We have observed more detailed internal structure of the tissue by the photoacoustic imaging.

In addition, we also utilized this photoacoustic micro-scopy to image blood vessels inside the submucosa. The dissected stomach wall after imaging by photoacoustic microscopy is shown in Figure 5A. The vessels under submucosa after imaging by photoacoustic microscopy is shown in Figure 5B. We very clearly saw the artery vessels and vein vessels.

In order to realize the photoacoustic depth imaging of the tumor, we designed a labeled tumor model based on ICG dye, wherein 0.1 mL ICG gelatin solution with a concentration of 129 μM/mL was injected into the stomach submucosa at 2-3 mm deep. After the ICG-gel mixture was injected into the tissue and coagulated for 20 minutes, the C-scan imaging at different depths was completed. When doing the scanning, the output wavelength of the laser was tuned to 700 nm because is the absorption peak of the ICG dye with molar extinction coefficient of 1.1×10^5 cm^{-1}/M in water. This will improve the signal: noise ratio of the image.

Some diffusion phenomena of the ICG resolution

Figure 4 Compared photoacoustic image with pathological photograph stained by hematoxylin-eosin staining for mucosa and submucosa of the tissue.

Figure 5 Photograph of the dissected stomach wall after imaging by photoacoustic microscopy (A) and photoacoustic imaging under surface of the tissue of 500 μm (red arrow indicate vessels) (B).

Figure 6 Photoacoustic imaging with laser wavelength of 700 nm for contrast agent with concentration of 12.9 μM/mL in depth of 400 μm (A) and 2400 μm (B).

at the mucosal site of 400 μm was observed (Figure 6A). The ICG dye may have combined with blood in the vessels, so some tubular structures were observed. At a depth of 2400 μm, the ICG labeled tumor model was still visible (Figure 6B). This indicated that we can achieve deeper detection.

DISCUSSION

Ultrasound uses high-frequency sound waves to produce images of the organs and structures inside the body such as ovaries, uterus, liver, gallbladder, pancreas, or aorta. EUS combines endoscopy and ultrasound in order to obtain images and information about the digestive tract and the surrounding tissue and organs. EUS can obtain information about the layers of the intestinal wall as well as adjacent areas such as lymph nodes and the blood vessels. EUS provides doctors with more information than other imaging tests by providing detailed images of the digestive tract. Although contrast-enhanced

ultrasonic techniques[13,14] such as Doppler ultrasound[15] are able to image blood vessels, the resolution of these imaging techniques are much lower than that of PAM, which has recently achieved lateral resolution up to 15 mm[16]. Additionally, photoacoustic imaging provides functional information with endogenous contrast and with the aid of an exogenous contrast agent. Therefore, photoacoustic endoscopy based on photoacoustic tomography and EUS, which has achieved spatially coincident photoacoustic and ultrasonic imaging, provides unprecedented information and promotes morphologic and functional understanding of the gastrointestinal tract imaging[17]. But the research on tissue structure based on photoacoustic imaging technique has not been published yet. This paper reported that utilizing AR-PAM to image layered tissue in the stomach wall and blood vessels under the mucosa in s without any contrasting agents. In the next step, this acoustic resolution photoacoustic microscopy will combine with endoscopy and minimize the photoacoustic probe to make a micro-photoacoustic endoscopy for obtaining morphologic and functional information with higher resolution and depth *in vivo*.

In conclusion, we have presented a fiber illumination based acoustic resolution photoacoustic microscopy method. We have realized an axial resolution of 25 μm and a lateral resolution of 50 μm in its focal zone range of 500 μm. This system was utilized to image layered stomach wall tissue and blood vessels under the mucosa without any contrasting agents. Using ICG dye enhance a tumor model, the tumor model was imaged to depths of 2400 μm with very high contrast. The photoacoustic microscopy has the ability to image layered tissue with high resolution and high contrast.

<div style="background:#E8820C; color:white; padding:4px;">

ARTICLE HIGHLIGHTS

</div>

Research background

When checking submucosal tumors, traditional methods (such as standard optical stomach endoscopy, capsule optical endoscopy and push-and-pull enteroscopy together with barium contrast X-ray) can't provide accurate information. Computed tomography and magnetic resonance imaging are often lower in resolution and contrast. Optimal endoscopic ultrasound (EUS) imaging of stomach submucosal tumors (SMT) needs submersion of the tumor under water. However, benign SMTs, for example the submucosal inflammation, cannot be distinguished endosonographically. Therefore, it is still a challenge for EUS to detect early-phase tumor *in situ*. Our team aimed to demonstrate that photoacoustic imaging is able to image layered tissue and blood vessels under the surface of the tissue. In the future, we can design and develop a photoacoustic endoscopic imaging system to image deeper tissues with higher resolution for diagnosis and treatment.

Research motivation

To image layered tissue and blood vessels in layered tissue.

Research objectives

Our aim is to demonstrate that photoacoustic imaging can detect the structure of layered tissue and blood vessels beneath the surface of the tissue.

Research methods

Our team established the acoustic resolution photoacoustic microscopy (AR-PAM) system for stomach wall structure imaging. Photoacoustic microimaging

of the stomach wall structure was compared to an HE pathology image to verify the structure, vessels, and vessel direction.

Research results

As a result, we have established a fiber illumination based acoustic resolution photoacoustic microscopy method. The imaging ability has an axial resolution of 25 μm and a lateral resolution of 50 μm in its focal zone range of 500 μm. We have observed more detailed internal structure of the tissue from AR-PAM imaging. We also utilized this photoacoustic microscopy to image blood vessels inside the submucosa. By using ICG dye enhance a tumor model in the submucosa, the images obtained at a depth of 2400 μm depth had very high contrast.

Research conclusions

In this study, we have established a fiber illumination based acoustic resolution photoacoustic microscopy. Layer-by-layer imaging of the stomach tissue and blood vessels under stomach mucosa were obtained. By using ICG dye to enhance the tumor model, high contrast images at a depth of 2400 μm tissue were obtained. This proved that photoacoustic microscopy has the ability to image layered tissue and deep tissue targets with high resolution and high contrast. In the near future, this technique combined with endoscopy will supply a simple tool for physicians to see differences in layered tissues and imaging of tumor angiogenesis in submucosa.

Research perspectives

This technique combined with endoscopy will supply a simple tool to visualize layered tissues and tumor angiogenesis in submucosa. Scientific research should aim at solving practical problems in clinical practice. Innovative scientific research is demonstrated in the ability to effectively solve the difficult problems doctors encounter in clinical practice.

REFERENCES

1   **Chak A**. EUS in submucosal tumors. *Gastrointest Endosc* 2002; **56**: S43-S48 [PMID: 12297748 DOI: 10.1016/S0016-5107(02)70085-0]

2   **Hsu CC**, Chen JJ, Changchien CS. Endoscopic features of metastatic tumors in the upper gastrointestinal tract. *Endoscopy* 1996; **28**: 249-253 [PMID: 8739742 DOI: 10.1055/s-2007-1005437]

3   **Hu S**, Wang LV. Photoacoustic imaging and characterization of the microvasculature. *J Biomed Opt* 2010; **15**: 011101 [PMID: 20210427 DOI: 10.1117/1.3281673]

4   **Shim CS**, Jung IS. Endoscopic removal of submucosal tumors: preprocedure diagnosis, technical options, and results. *Endoscopy* 2005; **37**: 646-654 [PMID: 16010609 DOI: 10.1055/s-2005-861477]

5   **Han S**, Hsu A, Wassef WY. An update in the endoscopic management of gastric cancer. *Curr Opin Gastroenterol* 2016; **32**: 492-500 [PMID: 27662061 DOI: 10.1097/MOG.0000000000000318]

6   **Ding Z**, Qiu J, Shen Y, Chen Z, Bao W. Lens-free all-fiber probe with an optimized output beam for optical coherence tomography. *Opt Lett* 2017; **42**: 2814-2817 [PMID: 28708176 DOI: 10.1364/OL.42.002814]

7   **Karia K**, Kahaleh M. A Review of Probe-Based Confocal Laser Endomicroscopy for Pancreaticobiliary Disease. *Clin Endosc* 2016; **49**: 462-466 [PMID: 27642847 DOI: 10.5946/ce.2016.086]

8   **Zhang HF**, Maslov K, Stoica G, Wang LV. Functional photoacoustic microscopy for high-resolution and noninvasive in vivo imaging. *Nat Biotechnol* 2006; **24**: 848-851 [PMID: 16823374 DOI: 10.1038/nbt1220]

9   **Maslov K**, Zhang HF, Hu S, Wang LV. Optical-resolution photoacoustic microscopy for in vivo imaging of single capillaries. *Opt Lett* 2008; **33**: 929-931 [PMID: 18451942 DOI: 10.1364/OL.33.000929]

10   **Brecht HP,** Su R, Fronheiser M, Ermilov SA, Conjusteau A, Liopo A, Motamedi M, Oraevsky AA. Optoacoustic 3D whole-body tomography: experiments in nude mice. *Proc SPIE* 2009; **7177**: 71770E [DOI: 10.1117/12.812319]

11   **Wang X**, Xie X, Ku G, Wang LV, Stoica G. Noninvasive imaging

of hemoglobin concentration and oxygenation in the rat brain using high-resolution photoacoustic tomography. *J Biomed Opt* 2006; **11**: 024015 [PMID: 16674205 DOI: 10.1117/1.2192804]

12   **Wang Y**, Hu S, Maslov K, Zhang Y, Xia Y, Wang LV. In vivo integrated photoacoustic and confocal microscopy of hemoglobin oxygen saturation and oxygen partial pressure. *Opt Lett* 2011; **36**: 1029-1031 [PMID: 21478972 DOI: 10.1364/OL.36.001029]

13   **Ignee A**, Jenssen C, Hocke M, Dong Y, Wang WP, Cui XW, Woenckhaus M, Iordache S, Saftoiu A, Schuessler G, Dietrich CF. Contrast-enhanced (endoscopic) ultrasound and endoscopic ultrasound elastography in gastrointestinal stromal tumors. *Endosc Ultrasound* 2017; **6**: 55-60 [PMID: 28218202 DOI: 10.4103/2303-9 027.200216]

14   **Dong Y**, Wang WP, Mao F, Ji ZB, Huang BJ. Application of imaging fusion combining contrast-enhanced ultrasound and magnetic resonance imaging in detection of hepatic cellular carcinomas undetectable by conventional ultrasound. *J Gastroenterol Hepatol* 2016; **31**: 822-828 [PMID: 26480287 DOI: 10.1111/jgh.13202]

15   **Xuan JW**, Bygrave M, Jiang H, Valiyeva F, Dunmore-Buyze J, Holdsworth DW, Izawa JI, Bauman G, Moussa M, Winter SF, Greenberg NM, Chin JL, Drangova M, Fenster A, Lacefield JC. Functional neoangiogenesis imaging of genetically engineered mouse prostate cancer using three-dimensional power Doppler ultrasound. *Cancer Res* 2007; **67**: 2830-2839 [PMID: 17363606 DOI: 10.1158/0008-5472.CAN-06-3944]

16   **Xing W**, Wang L, Maslov K, Wang LV. Integrated optical- and acoustic-resolution photoacoustic microscopy based on an optical fiber bundle. *Opt Lett* 2013; **38**: 52-54 [PMID: 23282835 DOI: 10.1364/OL.38.000052]

17   **Yang JM**, Favazza C, Chen R, Yao J, Cai X, Maslov K, Zhou Q, Shung KK, Wang LV. Simultaneous functional photoacoustic and ultrasonic endoscopy of internal organs in vivo. *Nat Med* 2012; **18**: 1297-1302 [PMID: 22797808 DOI: 10.1038/nm.2823]

ORIGINAL ARTICLE

Basic Study

Clinical correlation of B7-H3 and B3GALT4 with the prognosis of colorectal cancer

Ting Zhang, Fang Wang, Jing-Yi Wu, Zhi-Chao Qiu, Yan Wang, Fen Liu, Xiao-Song Ge, Xiao-Wei Qi, Yong Mao, Dong Hua

Ting Zhang, Fen Liu, Dong Hua, Institute of Cancer, Affiliated Hospital of Jiangnan University, Wuxi 214062, Jiangsu Province, China

Fang Wang, Jing-Yi Wu, Zhi-Chao Qiu, Yan Wang, Xiao-Song Ge, Yong Mao, Dong Hua, Department of Oncology, Affiliated Hospital of Jiangnan University, Wuxi 214062, Jiangsu Province, China

Fang Wang, Jing-Yi Wu, Zhi-Chao Qiu, Yan Wang, Wuxi School of Medicine, Jiangnan University, Wuxi 214122, Jiangsu Province, China

Xiao-Wei Qi, Department of Pathology, Affiliated Hospital of Jiangnan University, Wuxi 214062, Jiangsu Province, China

ORCID number: Ting Zhang (0000-0001-6859-6400); Fang Wang (0000-0002-8821-6695); Jing-Yi Wu (0000-0002-9219 -2382); Zhi-Chao Qiu (0000-0003-0837-6144); Yan Wang (0000-0002-6310-0987); Fen Liu (0000-0003-1891-6992); Xiao-Song Ge (0000-0002-4180-5900); Xiao-Wei Qi (0000-0001-7761-5095); Yong Mao (0000-0001-5507-8292); Dong Hua (0000-0001-5569-1708).

Abstract

AIM

To investigate the expression and clinical significance of B7 homolog 3 (B7-H3) and β-1,3-galactosyltransferase-4 (B3GALT4) in colorectal cancer (CRC) patients.

METHODS

Using tissue microarray, we identified the expression of B7-H3 and B3GALT4 in 223 CRC patient samples by immunohistochemistry and evaluated the possible correlation between B7-H3 and B3GALT4 and clinical

outcomes. Further, the mRNA and protein expression were identified to establish the regulatory relationship of B7-H3 with B3GALT4 *in vitro*.

RESULTS

A significant positive correlation between B7-H3 and B3GALT4 was observed in CRC specimens (r = 0.219, P = 0.001). High expression of B7-H3 was identified as a significant independent predictor of poor overall survival (OS) [hazard ratio (HR) = 1.781; 95%CI: 1.027-3.089; P = 0.040]. Moreover, high expression of B3GALT4 was also recognized as an independent predictor of inferior OS (HR = 1.597; 95%CI: 1.007-2.533; P = 0.047). Additionally, CRC patients expressing both high B7-H3 and high B3GALT4 contributed to a significant decrease in OS (HR = 2.283; 95%CI: 1.289-4.042; P = 0.005). In CRC cell lines with stable expression of high B7-H3, the mRNA and protein expressions of B3GALT4 were significantly upregulated. Similarly, the expression of B3GALT4 was significantly reduced when expression of B7-H3 was knocked down.

CONCLUSION

The expression of B3GALT4 in CRC is positively correlated with B7-H3 expression *in vitro*. B7-H3/B3GLAT4 may be used as dual prognostic biomarkers for CRC.

Core tip: The present study for the first time revealed the expression of β-1,3-galactosyltransferase-4 (B3GALT4) in colorectal cancer (CRC) and its correlation with B7 homolog 3 (B7-H3) *in vitro*. Overall, the findings of the present study suggest that B7-H3 and B3GALT4 are novel prognostic biomarkers for CRC and highlight the significance of both B7-H3 and B3GALT4 as promising therapeutic targets for CRC. Thus, here we present our preliminary work on the relationship of the immune function and glycosylation of tumor-associated protein in CRC.

INTRODUCTION

Colorectal cancer (CRC) is the most prevalent gastrointestinal tract malignancy worldwide among both men and women. Overall CRC incidence and mortality rates have been declining over the recent decades, yet CRC ranks third overall among other malignancies in men and women[1]. In China, there were approximately 376300 new cases and 191000 potential deaths for CRC in 2015[2]. Despite several therapeutic advancements including surgical resection, neoadjuvant chemoradiotherapy, and targeted therapy, the prognosis of CRC patients remains relatively poor. With unprecedented survival benefits in selected patients, immunotherapy has become a promising treatment strategy and continuous progress has been made with immunomodulatory agents that target immune system checkpoints such as anti-programmed death 1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4)[3].

B7 homolog 3 (B7-H3), also known as CD276, is a type I transmembrane glycoprotein that belongs to the B7/CD28 immunoglobulin superfamily. B7-H3 mRNA is widely expressed on many tissues and cell types. However, B7-H3 protein is not constitutively expressed on T-cells, natural killer cells and antigen-presenting cells, and its expression can be induced on immune cells. While limited expression has been noticed on normal tissues, overexpression is observed in a majority of human malignancies including CRC[4] and has been correlated with the poor prognosis of patients[5]. Moreover, B7-H3 has been suggested to promote tumor growth, migration, invasion, and metastasis. Thus, it may be a potential therapeutic target for active immunotherapy.

The β-1,3-galactosyltransferase-4 (B3GALT4) gene belongs to the β-1,3-galactosyltransferase (β3GalT) gene family, which encodes type II membrane-bound glycoproteins and is located in the centromeric segment of the human MHC class II region[6]. This gene is abundantly expressed in human organs and tissues, predominantly in the brain and involved in GM1/GD1 ganglioside synthesis[7]. The β3GalT family plays an essential role in the O-glycosylation process. The surface of cancer cells express glycoproteins, which are rich in O-glycosylation domains[8]. Thus, the family may be closely related to the tumor. Besides, Seko *et al*[9] had confirmed that B3GALT4 could be used as a novel biomarker for the diagnosis of gynecological cancers. However, there are insufficient reports about the correlation between B3GALT4 and CRC.

Therefore, the present study aimed to investigate the clinical correlation of B7-H3 and B3GALT4 with CRC. Further, correlation between the expression of B7-H3 and B3GALT4 was evaluated to determine their prognostic significance in CRC.

MATERIALS AND METHODS

Patients and tissue samples

The medical records of patients who received a histopathological diagnosis and underwent surgery for CRC at Affiliated Hospital of Jiangnan University between June 2008 and December 2011 were retrieved. A total

of 223 formalin-fixed paraffin-embedded CRC tissue samples were included in the study. These patients did not receive radiotherapy or chemotherapy before the surgery. Only histologically confirmed cases were included in the study. However, patients who received chemo- or radiotherapy before surgery and cases with incomplete clinical data were excluded from the study. The study was approved by the Medical Ethics Committee of Affiliated Hospital of Jiangnan University, and written informed consent was obtained from all patients. All the patients were followed up by telephone up to October 31, 2017, to obtain the survival data. The median follow-up was 79 mo (range, 6-114 mo).

Tissue microarray preparation

Two experienced pathologists examined the section stained with hematoxylin and eosin and marked the carcinoma sites in the corresponding paraffin block. Using a manual tissue microarrayer (Quick-Ray, UNITMA, Seoul, Korea), tissue cylinders with a 1.0 mm diameter were punched from representative tissue areas of each donor tissue block and implanted into the hole of the premade recipient paraffin block (UNITMA, Seoul, Korea). The tissue microarray paraffin block was then constructed and cut into 4 μm thick continuous sections, which were attached to anti-dewaxing slides and stored at room temperature.

Immunochemistry

The tissue paraffin blocks were serially sectioned into a 4 μm thickness, dewaxed in xylene, and hydrated in an ethanol gradient. Antigen retrieval was performed by heating the tissue sections at 100 ℃ for 30 min in citrate buffer. Moreover, endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 10 min. Subsequently, the sections were incubated in 5% bovine serum albumin at room temperature for 30 min. Primary antibodies, mouse anti-human B7-H3 monoclonal antibody (1:200, Santa Cruz, Dallas, TX, United States) and rabbit anti-human B3GALT4 monoclonal antibody (1:50, Abcam, Cambridge, MA, United States) respectively were added drop-wise followed by overnight incubation at 4 ℃. The slides were washed and incubated with a horseradish peroxidase-conjugated secondary antibody for 30 min. The immunostaining was carried out by staining with 3,3'-diaminobenzidine tetrahydrochloride solution (GTVisionII Immunohistochemistry Detection Kit for Rabbit/Mouse, Gene Tech, Shanghai, China), counter-stained with hematoxylin, dehydrated, and mounted. Sections were examined under a microscope.

Evaluation of immunochemistry staining

Two independent pathologists performed a blinded manner review of the sections. Both the intensity and extent of immunological staining were analyzed semi-quantitatively. According to the percentage of positively stained cells, the sections were graded by five levels: 0 (≤ 5%), 1 (6%-25%), 2 (26%-50%), 3 (51%-75%) and 4 (> 76%). The staining intensity was scored similarly, with 0 used for negative staining, 1 for weakly positive, 2 for moderately positive and 3 for strongly positive. The scores for the percentage of positive cells and the staining intensity were multiplied to generate an immune-reactive score for each specimen (0-12). Based on the total score, scores of 0-3 were considered as the low expression group, and scores of 4-12 were defined as the high expression group.

Cell lines and culture

The two human CRC cell lines, SW480 and Caco-2, which exhibited different expression levels of B7-H3 were maintained in our lab. We constructed SW480 cells that expressed a high level of B7-H3 (SW480-B7-H3) by transfection with an overexpression plasmid, and Caco-2 cells were stably transfected with a B7-H3 shRNA (Caco-2-shB7-H3). Cells transfected with a mock vector were used as negative controls (SW480-NC and Caco-2-shNC). SW480-NC and SW480-B7-H3 were cultured in L-15 medium (HyClone GE Healthcare Life Sciences, South Logan, UT, United States). Caco-2-NC and Caco-2-shB7-H3 were maintained in RPMI-1640 medium (the same as above). All the media were supplemented with 10% fetal bovine serum (Clark Bioscience, Houston, TX, United States). The cells were incubated at 37 ℃ in a humidified atmosphere with 5% CO_2.

RNA extraction and cDNA synthesis

Total RNA was extracted from 1×10^6 cells using TRIzol (Invitrogen, Carlsbad, CA, United States), according to the manufacturer's protocol. A NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, United States) was used to estimate the purity and concentration of total RNA by measuring the optical density. The first strand cDNA was generated from 1 μg RNA using the HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) following the manufacturer's instructions.

Real-time quantitative polymerase chain reaction

The expression levels of B7-H3 and B3GALT4 were detected using the QuantiNova™ SYBR Green PCR Kit (QIAGEN, Hilden, Germany) on ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, United States). β-actin was used as an internal control for normalization of the expression data. The first strand cDNA was amplified in a total 20 μL PCR reaction mixture: 10 μL 2 x QuantiNova SYBR Green PCR Master Mix, 0.1 μL QN ROX Reference Dye, 0.4 μL of each specific primer set, 2 μL cDNA and ddH2O added to 20 μL. The sequences of primers were as follows: β-actin 5'-CATGTACGTTGCTATCCAGGC-3' (sense), 5'-CTCCTTAATGTCACGCACGAT-3' (antisense); B7-H3 5'-AGCACTGTGGTTCTGCCTCACA-3' (sense), 5'-CACCAGCTGTTTGGTATCTGTCAG-3' (antisense); B3GALT4 5'-ACTCCTACCGCAACCTCA-3' (sense), 5'-CACATCATCGTCCGTCTT-3' (antisense). Each sample and internal control gene were run in triplicate, using an amplification condition of denaturation at 95 ℃ for

2 min, followed by 40 cycles of 95 ℃ for 5 s and 60 ℃ for 10 s. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of the target genes.

Western blotting

Total proteins were extracted from 1×10^6 cells by using ice-cold RIPA lysis buffer containing a cocktail of protease inhibitors (KeyGEN BioTECH, China) for 30 min. The concentrations of whole cell proteins were determined using a BCA Protein Assay Kit (CWBio, Beijing, China) and adjusted to the same concentration. For western blotting assay, the proteins of equal quantity were separated by 10% SDS-PAGE and then transferred onto a PVDF membrane (Merck Millipore, Germany). After blocking with 5% nonfat dry milk for 1 h at room temperature, the membranes were incubated with the primary antibodies at a concentration of 1:1000 at 4 ℃ overnight. The antibodies included rabbit anti-human B7-H3 monoclonal antibody (Abcam, Cambridge, MA, United States), rabbit anti-human B3GALT4 monoclonal antibody (Abcam, Cambridge, MA, United States) and mouse anti-human β-actin monoclonal antibody (Beyotime, Nantong, China). After washing with TBST (TBS with 0.1% Tween), the membranes were incubated with a corresponding goat-anti-mouse or goat-anti-rabbit IgG-horseradish peroxidase secondary antibody (1:1000, Beyotime, Nantong, China) for 1 h at room temperature. The membranes were finally treated with enhanced chemiluminescence (ECL) assay reagents (absin, Shanghai, China) and the immunoreactive bands were visualized using the ChemiDoc™ XRS+ system with Image Lab™ Software (Bio-Rad, Hercules, CA, United States). Quantitative analysis was carried out with Quantity One (Bio-Rad, Hercules, CA, United States).

Statistical analysis

Statistical analysis of clinical data was performed using the software package SPSS 19.0 software (IBM, Chicago, IL, United States). Chi-square test analyzed the association between B7-H3 or B3GALT4 and the clinicopathological data. The non-parametric Spearman test evaluated the correlation of B7-H3 and B3GALT4 expression. Overall survival (OS) was plotted using the Kaplan-Meier method and was compared using the log-rank test. Multivariate survival analysis was performed using the Cox proportional hazard model. All the *in vitro* experiments were performed in triplicate. A non-paired *t*-test analyzed differences in mean values between groups. All data were analyzed using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, United States). A *P*-value less than 0.05 indicated a statistically significant difference.

RESULTS

Expression of B7-H3 and B3GALT4 and their relationship with clinicopathological features of CRC

The expression of B7-H3 and B3GALT4 was analyzed using immunochemistry in CRC tissue. Positive staining of B7-H3 was predominantly detected in the membrane and cytoplasm (Figure 1C), and the location of B3GALT4 expression was identified in the cytoplasm (Figure 1D). The positive rates of the B7-H3 and B3GALT4 expressions were 70.4% (157/223) and 54.7% (122/223), respectively. Further, a highly significant correlation between the positive expression of B7-H3 with the depth of tumor invasion (*P* = 0.049), distant metastasis (*P* = 0.034) and differentiation (*P* = 0.017) was observed. However, the expression of B3GALT4 had no significant correlation with clinicopathological parameters (Table 1).

Correlation between B7-H3 and B3GALT4 expression in CRC

Of the 223 CRC cases, 97 (43.5%) patients exhibited higher expression of both B7-H3 and B3GALT4, and 41 (18.4%) showed lower expression of both. Also, there were 85 (38.1%) cases with either B7-H3 low or B3GALT4 low. Spearmen's Correlation was used to examine the association of B7-H3 expression with B3GALT4 expression in CRC. The result showed that there was a highly significant positive correlation between B7-H3 and B3GALT4 expression (*r* = 0.219, *P* = 0.001, Table 2).

High expression of B7-H3 and B3GALT4 in patients with CRC is associated with poor OS

Notably, patients with high expression of B7-H3 had a significantly worse OS as compared to patients with low expression (*P* = 0.037) (Figure 2A). Similarly, the OS in the high B3GALT4 expression group was significantly inferior to that in the low expression group (*P* = 0.044) (Figure 2B). Moreover, a subgroup of patients with high expression of B7-H3 and high B3GALT4 exhibited a significantly worse prognosis as compared to patients with a low expression of B3GALT4 (*P* = 0.016) (Figure 2C). However, in a subgroup of patients with low expression of both B7-H3 and B3GALT4 prognosis of patients with CRC was unaffected (*P* = 0.061) (Figure 2D).

Furthermore, the univariate Cox proportional hazard model analysis was performed to evaluate the risk factors related to the prognosis of patients with CRC. As shown in Table 3, high expression of B7-H3, high expression of B3GALT4, high expression of both B7-H3 and B3GALT4, depth of tumor invasion (T3/4), lymph node metastasis (N1/2), distant metastasis, TNM stage (III/IV), neural invasion, and vascular invasion were correlated with the OS of patients with CRC. However, there was no correlation of OS with gender, age, tumor location, colon cancer site, mucinous adenocarcinoma, differentiation, and B7-H3 low expression group (Table 3). Besides, the multivariate analysis revealed that distant metastasis, TNM stage (III/IV), and vascular invasion were significant independent prognostic factors for OS of patients with CRC (Table 3).

Table 1 Expression of B7 homolog 3 and β-1,3-galactosyltransferase-4 in colorectal cancer patients and their correlation with clinicopathological parameters

Clinicopathological parameter	Case (*n*)	B7-H3 expression		*P* value	B3GALT4 expression		*P* value
		Low	High		Low	High	
Gender				0.616			0.213
Male	124	35	89		63	61	
Female	99	31	68		42	57	
Age (yr)				0.259			0.791
< 60	87	22	65		40	47	
≥ 60	136	44	92		65	71	
Tumor location				0.361			0.905
Colon	88	23	65		41	47	
Rectum	135	43	92		64	71	
Colon cancer site				0.119			0.352
Right-sided	37	7	30		20	17	
Left-sided	186	59	127		85	101	
Depth of tumor invasion				0.049			0.527
T1/2	64	25	39		28	36	
T3/4	159	41	118		77	82	
Lymph node metastasis				0.433			0.145
N0	116	37	79		60	56	
N1/2	107	29	78		45	62	
Distant metastasis				0.034			0.425
Yes	16	1	15		6	10	
No	207	65	142		99	108	
TNM stage				0.297			0.068
I / II	113	37	76		60	53	
III / IV	110	29	81		45	65	
Neural invasion				0.146			0.683
Yes	32	6	26		14	18	
No	191	60	131		91	100	
Vascular invasion				0.079			0.585
Yes	35	6	29		15	20	
No	188	60	128		90	98	
Mucinous adenocarcinoma				0.579			0.506
Yes	20	7	13		8	12	
No	203	59	144		97	106	
Differentiation				0.017			0.186
Poor	73	14	59		39	34	
Moderate/well	150	52	98		66	84	

B7-H3: B7 homolog 3; B3GALT4: β-1,3-galactosyltransferase-4.

Table 2 Correlation between B7 homolog 3 and β-1,3-galactosyltransferase-4 expression in colorectal cancer

B3GALT4 expression	B7-H3 expression		*r*	*P* value
	Low	High		
Low	41	60	0.219	0.001
High	25	97		

B7-H3: B7 homolog 3; B3GALT4: β-1,3-galactosyltransferase-4.

Correlation between B7-H3 and B3GALT4 in CRC cell lines

We further investigated the relationship between expression of B7-H3 and B3GALT4 *in vitro*. The mRNA and protein expression was detected in the CRC cell lines with different B7-H3 expression levels, which were maintained in our lab. Both the mRNA and protein expression of B7-H3 and B3GALT4 were significantly upregulated in SW480-B7-H3 cell line expressing a high level of B7-H3 as compared to SW480-NC cell line. Similarly, as compared to Caco-2-shNC cell line,

Caco-2-shB7-H3 cell line with low expression of B7-H3 significantly downregulated the expression of B3GALT4. Therefore, B7-H3 and B3GALT4 had a positive correlation both in mRNA and protein expression *in vitro* (Figure 3).

DISCUSSION

CRC continues to be a major cause of cancer-related morbidity and mortality with over 1200000 cases diagnosed every year, and more than 600000 patients

Figure 1 The representative immunohistochemical images of B7 homolog 3 and β-1,3-galactosyltransferase-4 expression in colorectal cancer tissues.
A: Low expression of B7 homolog 3 (B7-H3); B: Low expression of β-1,3-galactosyltransferase-4 (B3GALT4); C: High expression of B7-H3; D: High expression of B3GALT4 (100 ×). The images below show the magnification of each zone (400 ×). A and B are from one patient; C and D are from another patient.

Figure 2 Kaplan-Meier survival curves of the 223 patients with colorectal cancer expressing B7 homolog 3 and β-1,3-galactosyltransferase-4. A: Patients with low expression of B7 homolog 3 (B7-H3) *vs* high expression of B7-H3; B: Patients with low expression of β-1,3-galactosyltransferase-4 (B3GALT4) *vs* high expression of B3GALT4; C: Subgroup of patients with high expression of B7-H3 and low expression of B3GALT4 *vs* high expression of B3GALT4; D: Subgroup of patients with low expression of B7-H3 and high expression of B3GALT4 *vs* low expression of B3GALT4.

succumb to the disease worldwide. Moreover, five-year survival rates for stage I CRC was more than 90% where as for stage IV was 10%[10]. Given the poor prognosis of CRC, the search for novel diagnostic and

Table 3 Univariate and multivariate Cox proportional hazards analyses of overall survival in patients with colorectal cancer

Clinical parameter	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Gender (male *vs* female)	0.999 (0.637-1.566)	0.995		
Age (≥ 60 *vs* < 60) (yr)	0.951 (0.603-1.500)	0.829		
Tumor location (rectum *vs* colon)	1.351 (0.863-2.117)	0.188		
Colon cancer site (left-sided *vs* right-sided)	0.970 (0.534-1.760)	0.919		
Depth of tumor invasion (T3/4 *vs* T1/2)	2.522 (1.362-4.670)	0.003	1.807 (0.949-3.443)	0.072
Lymph node metastasis (N1/2 *vs* N0)	2.922 (1.801-4.739)	0.000	0.322 (0.077-1.341)	0.119
Distant metastasis (yes *vs* no)	9.353 (5.206-16.804)	0.000	4.635 (2.224-9.660)	0.000
TNM stage (Ⅲ/Ⅳ *vs* Ⅰ/Ⅱ)	3.497 (2.114-5.783)	0.000	7.490 (1.636-34.280)	0.009
Neural invasion (yes *vs* no)	2.240 (1.320-3.802)	0.003	0.800 (0.410-1.561)	0.514
Vascular invasion (yes *vs* no)	2.531 (1.529-4.188)	0.000	1.962 (1.082-3.558)	0.026
Mucinous adenocarcinoma (yes *vs* no)	1.063 (0.488-2.314)	0.877		
Differentiation (poor *vs* moderate/well)	0.799 (0.504-1.267)	0.340		
B7-H3 (high *vs* low)	1.781 (1.027-3.089)	0.040	1.289 (0.721-2.307)	0.392
B3GALT4 (high *vs* low)	1.597 (1.007-2.533)	0.047	1.209 (0.721-2.307)	0.392
B7-H3 and B3GALT4 (high *vs* low)	0.737 (0.552-0.983)	0.038	1.153 (0.845-1.572)	0.369
B7-H3 high (B3GALT4 high *vs* low)	2.283 (1.289-4.042)	0.005		
B7-H3 low (B3GALT4 high *vs* low)	0.321 (0.091-1.127)	0.076		

B7-H3: B7 homolog 3; B3GALT4: β-1,3-galactosyltransferase-4.

prognostic biomarkers is highly desirable to prevent CRC-related deaths.

Immune checkpoint signaling pathways are most frequently modulated in cancer to inhibit the nascent anti-tumor immune response. Checkpoint antibody inhibitors, such as CTLA-4 and PD-1/PD-L1 are the most extensively studied novel class of inhibitors. Currently, drugs inhibiting these pathways are employed for a wide variety of malignancies and have demonstrated durable anti-tumor activities in a subset of cancer patients[11,12]. Furthermore, investigations on new inhibitory pathways are also undergoing, and drugs are being investigated including those targeting B7-H3[13]. As an immune checkpoint protein, B7-H3 has been recognized as an immunoregulatory molecule for T cell activation, proliferation, and cytokine production[14]. Thus far, overexpression of B7-H3 has been reported in several different cancer types and the expression level of B7-H3 correlated with tumor growth, invasion, metastasis, malignant stage, and recurrence rate[15]. Furthermore, several groups have generated anti-B7-H3 antibodies and reported overall suppression of tumor growth *in vitro* and *in vivo*[16]. Therefore, it is expected that B7-H3 plays a crucial role in cancer diagnosis and treatment. The present study revealed that B7-H3 was highly expressed in CRC tissue (70.4%) and significantly associated with the depth of tumor invasion (*P* = 0.049), distant metastasis (*P* = 0.034) and differentiation (*P* = 0.017). These results were consistent with previous findings[17,18].

Furthermore, B7-H3 is a type I transmembrane glycoprotein and plays a role in immunosuppression. Chen *et al*[19] demonstrated that glycoprotein B7-H3 was involved in the pathological process and tumor growth of oral squamous cell carcinoma, and the glycans of B7-H3 from oral cancer cells comprised of terminal α-galactoses and more diverse N-glycan structures with higher fucosylation than that of healthy cells. In addition, the presence of a carbohydrate-lectin receptor interaction between the tumor-associated B7-H3 from oral cancer cells and immune cells was detected[19]. B3GALT4, encoding a UDP-galactose: β-N-acetylgalactosamine β-1,3-galactosyltransferase activity, belongs to the human β-3-galactosyltransferase gene family[7]. Galactosyltransferase (GT) of the glycosyltransferase family, is the most common Golgi "marker" enzyme, catalyzes the transfer of galactose to glycoprotein-bound acetylglucosamine. The enzyme is provided with one N-linked oligosaccharide and palmitate residues and plays a vital role in the process of O-glycosylation. Moreover, in patients suffering from ovarian and breast cancer, increased expression of GT enzyme activity has been reported[20]. Enzymatic activity of B3GALT4 and T5 in ovarian cancer tissues, and significant overexpression of B3GALT4/T5 mainly in stage I uterine corpus cancers, indicated that B3GALT4/T5 is a novel tumor marker for uterine corpus cancer and other gynecological cancers[9]. Consequently, B7-H3 and B3GALT4 were both correlated with glycosylation and tumor progession. However, there was a paucity of literature on the expression and association of B7-H3 and B3GALT4 in CRC. Therefore, the association of B7-H3 and B3GALT4 expression in CRC tissues and cell lines was investigated.

The present study revealed that high expression of B7-H3 or B3GALT4 reduced the OS rate of CRC patients independently (*P* = 0.037, *P* = 0.044, respectively). Furthermore, overexpression of both B7-H3 and B3GALT4 in CRC resulted in poorer prognosis (*P* = 0.016). Besides, there was a significant positive correlation between B7-H3 and B3GALT4 (*r* = 0.219, *P* = 0.001). Taken together, B7-H3 and B3GALT4 could serve as a novel, reliable prognostic marker for CRC. Furthermore, we detected that the expression of B3GALT4 was positively altered following B7-H3 expression *in vitro*.

Figure 3 The expression of B7 homolog 3 and β-1,3-galactosyltransferase-4 in colorectal cancer cell lines. A: Real-time quantitative polymerase chain reaction results of relative mRNA expression; B: Western blotting results for protein expression; C: Statistical analysis of protein expression ([a]$P < 0.05$, [b]$P < 0.01$).

Combined with the results of clinical samples, this study indicated that B7-H3 could positively regulate B3GALT4 at the mRNA as well as the protein level. However, the underlying mechanism of regulation of B7-H3 and B3GALT4 in CRC warrants further investigation.

In summary, the present study for the first time revealed the expression of B3GALT4 in CRC and its correlation with B7-H3 *in vitro*. Overall, the findings of the present study suggested that B7-H3 and B3GALT4 are novel prognostic biomarkers for CRC and highlight the significance of both B7-H3 and B3GALT4 as promising therapeutic targets for CRC. Thus, here we present our preliminary work on the relationship of the immune function and glycosylation of tumor-associated protein in CRC.

ARTICLE HIGHLIGHTS

Research background
Colorectal cancer (CRC) is the most prevalent gastrointestinal tract malignancy worldwide. The prognosis of CRC patients remains relatively poor. B7 homolog 3 (B7-H3) mRNA is widely expressed on many tissues and cell types. However, B7-H3 protein is not constitutively expressed on T-cells, natural killer cells and antigen-presenting cells. The β-1,3-galactosyltransferase-4 (B3GALT4), which belongs to β-1,3-galactosyltransferase (β3GalT) gene family is abundantly expressed in human organs and tissues, predominantly in brain and involved in

GM1/GD1 ganglioside synthesis. The β3GalT family may be closely related to the tumor.

Research motivation
There are insufficient reports about the correlation between B3GALT4 and CRC.

Research objectives
The aim of the present study is to investigate the clinical correlation of B7-H3 and B3GALT4 with CRC, and the correlation between the expression of B7-H3 and B3GALT4 was evaluated to determine their prognostic significance in CRC.

Research methods
The authors identified the expression of B7-H3 and B3GALT4 in 223 CRC patient samples by immunohistochemistry and evaluated the possible correlation between B7-H3 and B3GALT4 and clinical outcomes. The mRNA and protein expression were also identified to establish the regulatory relationship of B7-H3 with B3GALT4 *in vitro*.

Research results
A significant positive correlation between B7-H3 and B3GALT4 was observed in CRC specimens. High expression of B7-H3 was identified as a significant independent predictor of poor overall survival (OS). High expression of B3GALT4 was also recognized as an independent predictor of inferior OS. In CRC cell lines with the stable expression of high B7-H3, the mRNA and protein expressions of B3GALT4 were significantly upregulated. The expression of B3GALT4 was significantly reduced when expression of B7-H3 was knocked down.

Research conclusions

The expression of B3GALT4 in CRC and its positive correlation with B7-H3 *in vitro* was revealed, as well as B7-H3/B3GLAT4 as dual prognostic biomarkers for CRC.

Research perspectives

The present study suggested that B7-H3 and B3GALT4 are novel prognostic biomarkers for CRC, and the significance of both B7-H3 and B3GALT4 as promising therapeutic targets for CRC are highlighted.

REFERENCES

1   **Siegel RL**, Miller KD, Fedewa SA, Ahnen DJ, Meester RGS, Barzi A, Jemal A. Colorectal cancer statistics, 2017. *CA Cancer J Clin* 2017; **67**: 177-193 [PMID: 28248415 DOI: 10.3322/caac.21395]

2   **Chen W**, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. *CA Cancer J Clin* 2016; **66**: 115-132 [PMID: 26808342 DOI: 10.3322/caac.21338]

3   **Zhang Y**, Chen Z, Li J. The current status of treatment for colorectal cancer in China: A systematic review. *Medicine* (Baltimore) 2017; **96**: e8242 [PMID: 28984783 DOI: 10.1097/MD.0000000000008242]

4   **Castellanos JR**, Purvis IJ, Labak CM, Guda MR, Tsung AJ, Velpula KK, Asuthkar S. B7-H3 role in the immune landscape of cancer. *Am J Clin Exp Immunol* 2017; **6**: 66-75 [PMID: 28695059]

5   **Ye Z**, Zheng Z, Li X, Zhu Y, Zhong Z, Peng L, Wu Y. B7-H3 Overexpression Predicts Poor Survival of Cancer Patients: A Meta-Analysis. *Cell Physiol Biochem* 2016; **39**: 1568-1580 [PMID: 27626927 DOI: 10.1159/000447859]

6   **Shiina T**, Kikkawa E, Iwasaki H, Kaneko M, Narimatsu H, Sasaki K, Bahram S, Inoko H. The beta-1,3-galactosyltransferase-4 (B3GALT4) gene is located in the centromeric segment of the human MHC class II region. *Immunogenetics* 2000; **51**: 75-78 [PMID: 10663566 DOI: 10.1007/s002510050012]

7   **Amado M**, Almeida R, Carneiro F, Levery SB, Holmes EH, Nomoto M, Hollingsworth MA, Hassan H, Schwientek T, Nielsen PA, Bennett EP, Clausen H. A family of human beta3-galactosyltransferases. Characterization of four members of a UDP-galactose:beta-N-acetyl-glucosamine/beta-nacetyl-galactosamine beta-1,3-galactosyltransferase family. *J Biol Chem* 1998; **273**: 12770-12778 [PMID: 9582303 DOI: 10.1074/jbc.273.21.12770]

8   **Wandall HH**, Blixt O, Tarp MA, Pedersen JW, Bennett EP, Mandel U, Ragupathi G, Livingston PO, Hollingsworth MA, Taylor-Papadimitriou J, Burchell J, Clausen H. Cancer biomarkers defined by autoantibody signatures to aberrant O-glycopeptide epitopes. *Cancer Res* 2010; **70**: 1306-1313 [PMID: 20124478 DOI: 10.1158/0008-5472.CAN-09-2893]

9   **Seko A**, Kataoka F, Aoki D, Sakamoto M, Nakamura T, Hatae M, Yonezawa S, Yamashita K. Beta1,3-galactosyltransferases-4/5 are novel tumor markers for gynecological cancers. *Tumour Biol* 2009; **30**: 43-50 [PMID: 19225246 DOI: 10.1159/000203129]

10 **Brenner H**, Kloor M, Pox CP. Colorectal cancer. *Lancet* 2014; **383**: 1490-1502 [PMID: 24225001 DOI: 10.1016/S0140-6736(13)61649-9]

11 **Seidel JA**, Otsuka A, Kabashima K. Anti-PD-1 and Anti-CTLA-4 Therapies in Cancer: Mechanisms of Action, Efficacy, and Limitations. *Front Oncol* 2018; **8**: 86 [PMID: 29644214 DOI: 10.3389/fonc.2018.00086]

12 **Abdo J**, Cornell DL, Mittal SK, Agrawal DK. Immunotherapy Plus Cryotherapy: Potential Augmented Abscopal Effect for Advanced Cancers. *Front Oncol* 2018; **8**: 85 [PMID: 29644213 DOI: 10.3389/fonc.2018.00085]

13 **Marin-Acevedo JA**, Dholaria B, Soyano AE, Knutson KL, Chumsri S, Lou Y. Next generation of immune checkpoint therapy in cancer: new developments and challenges. *J Hematol Oncol* 2018; **11**: 39 [PMID: 29544515 DOI: 10.1186/s13045-018-0582-8]

14 **Picarda E**, Ohaegbulam KC, Zang X. Molecular Pathways: Targeting B7-H3 (CD276) for Human Cancer Immunotherapy. *Clin Cancer Res* 2016; **22**: 3425-3431 [PMID: 27208063 DOI: 10.1158/1078-0432.CCR-15-2428]

15 **Li G**, Quan Y, Che F, Wang L. B7-H3 in tumors: friend or foe for tumor immunity? *Cancer Chemother Pharmacol* 2018; **81**: 245-253 [PMID: 29299639 DOI: 10.1007/s00280-017-3508-1]

16 **Shi J**, Zhang DL, Cui ZC, Wang HM. Preparation and application of a novel monoclonal antibody specific for human B7-H3. *Mol Med Rep* 2016; **14**: 943-948 [PMID: 27222007 DOI: 10.3892/mmr.2016.5288]

17 **Sun J**, Chen LJ, Zhang GB, Jiang JT, Zhu M, Tan Y, Wang HT, Lu BF, Zhang XG. Clinical significance and regulation of the costimulatory molecule B7-H3 in human colorectal carcinoma. *Cancer Immunol Immunother* 2010; **59**: 1163-1171 [PMID: 20333377 DOI: 10.1007/s00262-010-0841-1]

18 **Mao Y**, Sun J, Wang WP, Zhang XG, Hua D. Clinical significance of costimulatory molecule B7-H3 expression on CD3(+) T cells in colorectal carcinoma. *Chin Med J* (Engl) 2013; **126**: 3035-3038 [PMID: 23981607]

19 **Chen JT**, Chen CH, Ku KL, Hsiao M, Chiang CP, Hsu TL, Chen MH, Wong CH. Glycoprotein B7-H3 overexpression and aberrant glycosylation in oral cancer and immune response. *Proc Natl Acad Sci USA* 2015; **112**: 13057-13062 [PMID: 26438868 DOI: 10.1073/pnas.1516991112]

20 **Strous GJ**. Golgi and secreted galactosyltransferase. *CRC Crit Rev Biochem* 1986; **21**: 119-151 [PMID: 3093147 DOI: 10.3109/10409238609113610]

ORIGINAL ARTICLE

Retrospective Cohort Study

Favorable clinical outcome of nonalcoholic liver cirrhosis patients with coronary artery disease: A population-based study

Ming-Chang Tsai, Tzu-Wei Yang, Chi-Chih Wang, Yao-Tung Wang, Wen-Wei Sung, Ming-Hseng Tseng, Chun-Che Lin

Ming-Chang Tsai, Tzu-Wei Yang, Chi-Chih Wang, Chun-Che Lin, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Chung Shan Medical University Hospital, Taichung 402, Taiwan

Ming-Chang Tsai, Chi-Chih Wang, Yao-Tung Wang, Wen-Wei Sung, Chun-Che Lin, Institute of Medicine, Chung Shan Medical University, Taichung 402, Taiwan

Ming-Chang Tsai, Tzu-Wei Yang, Chi-Chih Wang, Wen-Wei Sung, Ming-Hseng Tseng, Chun-Che Lin, School of Medicine, Chung Shan Medical University, Taichung 402, Taiwan

Tzu-Wei Yang, Institute and Department of Biological Science and Technology, National Chiao Tung University, Hsinchu 300, Taiwan

Yao-Tung Wang, Division of Pulmonary Medicine, Chung Shan Medical University Hospital, Taichung 402, Taiwan

Wen-Wei Sung, Department of Urology, Chung Shan Medical University Hospital, Taichung 402, Taiwan

Ming-Hseng Tseng, Department of Medical Informatics, Chung Shan Medical University, Taichung 402, Taiwan

ORCID number: Ming-Chang Tsai (0000-0002-7687-2329); Tzu-Wei Yang (0000-0002-1522-8177); Chi-Chih Wang (0000-0002-8222-0503); Yao-Tung Wang (0000-0002-0300-0896); Wen-Wei Sung (0000-0002-2375-0029); Ming-Hseng Tseng (0000-0001-8868-1610); Chun-Che Lin (0000-0002-2474-6734).

Correspondence to: Chun-Che Lin, MD, PhD, Professor,

Division of Gastroenterology and Hepatology, Department of Internal Medicine, Chung Shan Medical University Hospital, No.110, Sec. 1, Jianguo N. Rd., South Dist., Taichung 402, Taiwan. forest65@csmu.edu.tw
Telephone: +886-4-24739595
Fax: +886-4-24739220

Abstract

AIM
To elucidate the prevalence and risk of mortality of nonalcoholic liver cirrhosis (LC) patients with coronary artery disease (CAD).

METHODS
The study cohort included newly diagnosed nonalcoholic LC patients age $\geq$ 40 years old without a diagnosis of CAD from 2006 until 2011 from a longitudinal health insurance database. The mean follow-up period for the study cohort was 1152 $\pm$ 633 d. The control cohort was matched by sex, age, residence, and index date. Hazard ratios (HRs) were calculated using the Cox proportional hazard model and the Kaplan-Meier method.

RESULTS
After exclusion, a total of 3409 newly diagnosed nonalcoholic cirrhotic patients were identified from one million samples from the health insurance database. We found that CAD (5.1% *vs* 17.4%) and hyperlipidemia (20.6% *vs* 24.1%) were less prevalent in nonalcoholic LC patients than in normal subjects (all $P < 0.001$), whereas other comorbidities exhibited an increased prevalence. Among the comorbidities, chronic kidney disease exhibited the highest risk for mortality (adjusted HR (AHR) = 1.76; 95%CI: 1.55-2.00, $P < 0.001$). Ascites or peritonitis exhibited the highest risk of mortality among nonalcoholic cirrhotic patients (AHR = 2.34; 95%CI: 2.06-2.65, $P < 0.001$). Finally, a total of 170 patients developed CAD after a diagnosis of nonalcoholic LC. The AHR of CAD in nonalcoholic LC patients was 0.56 (95%CI: 0.43-0.74, $P < 0.001$). The six-year survival rates for nonalcoholic LC patients with and without CAD were 52% and 50%, respectively ($P = 0.012$).

CONCLUSION
We conclude that CAD was less prevalent and associated with a reduced risk of mortality in nonalcoholic cirrhotic patients.

Key words: Nonalcoholic liver cirrhosis; Coronary artery disease; Population-based study

Core tip: Coronary artery disease (CAD) is less prevalent and associated with a reduced risk of mortality in nonalcoholic liver cirrhosis (LC) patients. Nonalcoholic LC patients with CAD exhibit an increased six-year survival rate compared to cirrhotic patients without CAD. The LC complication rates did not differ between nonalcoholic LC patients with and without CAD. Of note, nonalcoholic LC patients with ascites or peritonitis exhibited the highest risk of mortality among LC complications.

INTRODUCTION

Liver cirrhosis (LC) is the 14th leading cause of death in adults and accounted for one million deaths in 2010 worldwide[1]. In Taiwan, LC and chronic liver disease constituted the 8th most common cause of death in 2011[2]. These conditions contribute to numerous lethal complications and comorbidities that result in poor clinical outcomes. Variceal bleeding, systemic infection, hepatic encephalopathy, and hepatocellular carcinoma (HCC) are the primary complications of LC and causes of mortality[3,4]. In addition, LC is associated with systemic comorbidities, such as hemorrhagic stroke and digestive tract malignancies[5-7].

Controversy has emerged regarding the association between LC and coronary artery disease (CAD) in recent decades[7-17]. Several comorbidities of cirrhotic patients have been regarded as cardioprotective factors (*e.g.,* low blood pressure, coagulopathy, thrombocytopenia, malnutrition, and lower serum cholesterol levels)[8,18-20]. However, case-controlled studies have revealed conflicting results and an increased prevalence of CAD in LC patients compared to the normal population[7,9,17,21,22]. A different etiology with an increased proportion of alcoholic and nonalcoholic steatohepatitis (NASH)-related LC in Western populations may have contributed to this result. Furthermore, the application of interferon and nucleot(s)ide analogues for viral hepatitis aided in prolonging survival in recent decades[23,24]. Age-related risks associated with CAD also may have led to an increased prevalence of CAD.

There is a paucity of available data regarding the association between nonalcoholic LC and CAD. Taiwan is an endemic area of viral hepatitis where the majority of LC in patients is caused by hepatitis B (HBV) and hepatitis C (HCV) viral infections[25]. Hence, the nonalcoholic LC population in Taiwan is an ideal cohort in which the confounding effect of alcohol consumption

is minimized.

The aim of our study was to elucidate the association between nonalcoholic LC and CAD using a national population-based database in Taiwan. Additionally, we analyzed the prevalence, hazard ratio (HR), and survival for concomitant comorbidities and complications.

MATERIALS AND METHODS

Study population and design
This study was a nationwide retrospective longitudinal population-based cohort study based on the Taiwanese National Health Insurance research database (NHIRD). The National Health Insurance program has provided compulsory medical insurance for greater than 99% of the Taiwanese population since March 1, 1995. We identified all LC patients with a first-time diagnosis of LC using International Classification of Disease, 9th Revision, Clinical Modification (ICD-9-CM) codes (ICD-9-CM code: 571.5) for the period from 2004 to 2011. The following patients were excluded from the study: patients who had LC or CAD before the end of 2005, had LC after diagnosis of CAD from 2006 to 2011, were younger than 40 years old, had a diagnosis of alcoholic LC (ICD-9-CM code: 571.2) or biliary LC (ICD-9-CM code: 571.6), and were outpatients with less than two follow-up visits after the first visit. The control subjects were matched with the patients in the study cohort at a ratio of five to one (5 control subjects *per* case patient) in terms of sex, age (40-49, 50-59, 60-69, and > 69 years), residence, and entry year.

Other comorbidity data and the ICD-9-CM codes are summarized in Table Appendix 1. The diagnosis of comorbidities was defined as having three outpatient visits or one admission. Hyperlipidemia was defined as having a lipid profile with any one of the following factors: total cholesterol (TC) $\geq$ 2000 mg/L, low-density lipoprotein cholesterol (LDL-C) $\geq$ 1600 mg/L, or a triglyceride (TG) level $\geq$ 1500 mg/L. Complications of LC that occurred after enrollment included esophageal varices (EV) (ICD-9-CM codes: 456-456.21), EV with bleeding (ICD-9-CM codes: 456.0, 456.20) concomitant with ICD-9 procedure codes for ligation of EV or sclerotherapy, ascites (ICD-9-CM codes: 789.5 and 568.82) or peritonitis (ICD-9-CM codes: 567.xx), and hepatic encephalopathy (ICD-9-CM code: 572.2).

End point measurement
We followed up all the subjects enrolled from the index date to the end of 2011. The mean follow-up period for the newly diagnosed nonalcoholic LC cohort (3409 patients) was 1152 ± 633 d with a median of 1169 d, maximum of 2920 d, and minimum of 7 d. Patients with newly diagnosed CAD (one inpatient or three outpatient codes) were categorized based on the first incidence of CAD. The first-time diagnosis of CAD was identified as the primary end point, and the death of the subject served as the secondary end point. All-cause mortality was analyzed in both the study and control cohorts.

Statistical analysis
Microsoft SQL Server 2008 R2 (Microsoft Corporation, Redmond, WA, United States) was used to perform data processing. Statistical analyses were conducted using SPSS software 19.0 (SPSS, Inc., Chicago, IL, United States). Chi-square tests and analysis of variance (ANOVA) were used to analyze the demographic data, concomitant comorbidities, and complications of cirrhosis. A Cox proportional hazards model was developed to calculate the overall mortality HRs of all comorbidities and complications in cirrhotic patients. The results are expressed in unadjusted and adjusted HRs (AHRs) with 95% confidence intervals (CI). The six-year cumulative survival and survival curve were calculated using the Cox regression method and Kaplan-Meier method. A two-tailed *P* value less than or equal to 0.05 was considered statistically significant.

RESULTS

Primary outcome: CAD was less prevalent in patients with nonalcoholic LC
A total of 10142 cases of LC were retrieved from one million random samples in the NHIRD from 2004 to 2011. To ensure that only newly diagnosed LC cases were included, we excluded patients who met the following criteria: LC diagnosed before the end of 2005, CAD occurred before LC, alcoholic or biliary LC, and age younger than 40 years old. Finally, a total of 3409 newly diagnosed nonalcoholic LC patients between 2006 and 2011 were identified and included in the study (Figure 1). To select age-, sex-, residence-, and entry year-matched controls, we further excluded 173 cirrhotic patients to achieve 100% randomization. A total of 3236 patients with nonalcoholic LC and 16180 matched controls were analyzed for all demographic characteristics (Table 1). Patients with nonalcoholic LC were more likely to have comorbidities including hemorrhagic stroke, hypertension, heart failure (HF), diabetes mellitus (DM), chronic kidney disease (CKD), chronic obstructive pulmonary disease (COPD) (all *P* < 0.001), and ischemic stroke (*P* = 0.005). In contrast, hyperlipidemia was less prevalent in nonalcoholic LC patients (*P* < 0.001). During six years of follow-up, 165 (5.1%) patients in the nonalcoholic LC cohort and 2814 (17.4%) subjects in the control cohort developed CAD (*P* < 0.001).

Nonalcoholic LC patients with and without CAD
As shown in Table 2, we stratified nonalcoholic LC patients (*n* = 3409) into two groups according to the presence of CAD. Nonalcoholic LC patients with CAD were older ($\geq$ 60 years old), more likely female, and exhibited increased concomitant comorbidities of ischemic stroke, hypertension, HF, DM, CKD, hyperlipidemia, and COPD (all *P* < 0.001). When we compared the complications of liver cirrhosis between the two groups, nonalcoholic LC patients with CAD had fewer LC-related complications, including EV with bleeding, ascites or peritonitis, and hepatic encephalo-

Figure 1 Study design.

Table 1 Characteristics of non-alcoholic liver cirrhosis cohort and the matched control cohort *n* (%)			
Variable	Non-alcoholic LC patients, *n* = 3236	Control, *n* = 16180	*P* value
Sex			1.000
Male	2083 (64.4)	10415 (64.4)	
Female	1153 (35.6)	5765 (35.6)	
Age (yr)			1.000
40-49	565 (17.5)	2825 (17.5)	
50-59	885 (27.3)	4425 (27.3)	
60-69	766 (23.7)	3830 (23.7)	
> 69	1020 (31.5)	5100 (31.5)	
Residence			1.000
Metropolis	1834 (56.7)	9170 (56.7)	
General area	1362 (42.1)	6810 (42.1)	
Remote areas	40 (1.2)	200 (1.2)	
Coronary artery disease	165 (5.1)	2814 (17.4)	< 0.001
Cerebrovascular disease	413 (12.8)	1580 (9.8)	< 0.001
Hemorrhage	110 (3.4)	283 (1.7)	< 0.001
Ischemia	333 (10.3)	1413 (8.7)	0.005
Hypertension	1620 (50.1)	6672 (41.2)	< 0.001
Heart failure	252 (7.8)	972 (6.0)	< 0.001
Diabetes mellitus	1075 (33.2)	3141 (19.4)	< 0.001
Chronic kidney disease	616 (19.0)	1216 (7.5)	< 0.001
Hyperlipidemia	668 (20.6)	3903 (24.1)	< 0.001
Chronic obstructive pulmonary disease	534 (16.5)	1976 (12.2)	< 0.001

LC: Liver cirrhosis.

pathy (Supplementary Table 1). However, no significant differences were noted between the two groups.

Secondary outcome: Increased survival rate in the nonalcoholic LC with CAD group

We analyzed 3409 nonalcoholic LC patients and 16180 control subjects with a mean follow-up period of six years, and six-year survival rates were 50% and 85%, respectively ($P < 0.001$) (Figure 2A). When we compared nonalcoholic LC patients with and without CAD, the six-year survival rates were 52% and 50%, respectively ($P = 0.012$) (Figure 2B). The Cox regression proportional hazard model corresponding to each group also demonstrated a better survival rate for the nonalcoholic LC with CAD group than for those without CAD (AHR: 0.56; 95%CI: 0.43-0.74; $P < 0.001$) (Table 3).

HRs of mortality in LC patients with different comorbidities and complications

Table 3 presents the HRs of mortality in LC patients with different comorbidities. Increased HRs were observed for males, older patients, cerebrovascular disease (CVD), HF, DM, CKD and COPD. Among these factors, CKD exhibited the highest AHR of mortality (AHR: 1.76; 95%CI: 1.55-2.00). The AHRs of CAD, hypertension, and hyperlipidemia were reduced. Hyperlipidemia exhibited the lowest AHR of 0.52 (95%CI: 0.44-0.62).

When we analyzed the HRs of LC complications that occurred after enrollment, all complications exhibited increased values for both unadjusted HRs and AHRs. Ascites or peritonitis exhibited the highest AHR at 2.34 (95%CI: 2.06-2.65) (Supplementary Table 2).

Finally, we compared the effect of the number of comorbidities and complications on the survival rate in LC patients. Cirrhotic patients with one, two, or three comorbidities had HRs of 1.35, 1.72, and 1.95 (95%CI: 1.19-1.55, 1.47-2.00, and 1.57-2.43), respectively (Supplementary Table 3). However, the HRs for one, two, or t three LC complications were 3.56, 4.63, and 5.15 (95%CI: 3.14-4.03, 3.94-5.44, and 3.82-6.94), respectively (Supplementary Table 4).

Table 2 Characteristics of non-alcoholic liver cirrhosis patients with and without coronary artery disease *n* (%)

Variable	Non-alcoholic LC with CAD, *n* = 170	Non-alcoholic LC without CAD, *n* = 3239	*P* value
Sex			0.094
Male	99 (58.2)	2091 (64.6)	
Female	71 (41.8)	1148 (35.4)	
Age (yr)			< 0.001
40-49	18 (10.6)	554 (17.1)	
50-59	25 (14.7)	877 (27.1)	
60-69	46 (27.1)	751 (23.2)	
> 69	81 (47.6)	1057 (32.6)	
Residence			0.484
Metropolis	89 (52.4)	1830 (56.5)	
General area	79 (46.5)	1358 (41.9)	
Remote areas	2 (1.2)	51 (1.6)	
Cerebrovascular disease	46 (27.1)	400 (12.3)	< 0.001
Hemorrhage	9 (5.2)	103 (3.2)	0.132
Ischemia	37 (21.8)	327 (10.1)	< 0.001
Hypertension	139 (81.8)	1594 (49.2)	< 0.001
Heart failure	54 (31.8)	215 (6.6)	< 0.001
Diabetes mellitus	84 (49.4)	1051 (32.4)	< 0.001
Chronic renal failure	68 (40.0)	587 (18.1)	< 0.001
Hyperlipidemia	54 (31.8)	656 (20.3)	< 0.001
Chronic obstructive pulmonary disease	49 (28.8)	521 (16.1)	< 0.001

LC: Liver cirrhosis; CAD: Coronary artery disease.

DISCUSSION

In the present study, we observed a reduced prevalence of CAD and an increased survival rate for concomitant CAD in nonalcoholic LC patients. A lower prevalence of atherosclerosis and CAD was first described in an autopsy-based study in 1960[10]. A reduced prevalence of ischemic events was also reported for ischemic stroke[8,26]. However, conflicting data have been reported in some retrospective cohort and case-controlled studies[8,9,13,16,17,21,22]. First, the increased prevalence of CAD found in LC patients might be due to the different etiologies of LC in these Western population-based cohorts. An increased proportion of NASH and alcohol-related LC was noted compared to cohorts in viral hepatitis endemic areas[13,16,21,22]. NASH and alcohol consumption are generally associated with increased CAD risk factors. Nonetheless, the prevalence of CAD was reduced in end-stage liver disease patients assessed for liver transplantation who had alcoholic LC when compared to those with nonalcoholic LC[27]. Although moderate consumption of alcohol (< 30 g/d) has been reported as a protective factor against CAD[12], controversy still exists regarding heavy drinkers. In comparison, most subjects in our study had viral hepatitis-related LC. Few metabolic disorders were associated with steatohepatitis (such as hyperlipidemia), and no alcohol consumption was reported in our study cohort.

Second, lower blood pressure, better serum lipid profiles (including lower LDL-C, TC, and TG), and coagulopathy in LC patients were regarded as protective

factors against CAD[8,18-20,22]. However, viral hepatitis *per se* can influence vessel function and cause endothelial dysfunction, which is correlated with atherosclerotic disease progression[28]. A retrospective cohort study indicated that hepatitis C virus-infected subjects exhibited an increased risk for CAD, although better blood pressure and lipid profiles were observed in the population[29,30]. Consistent with previous reports, we observed a reduced prevalence of hyperlipidemia in cirrhotic patients. The proportion of comorbidities, including CVD, hypertension, HF, DM, CKD, hyperlipidemia, and COPD, was increased in the nonalcoholic LC with CAD patients[7]. The increase in these age-related comorbidities may be attributed to the older age in this study cohort. Third, CAD is an age-related disease. We hypothesize that better liver function may contribute to increased survival in the cirrhotic patients with CAD cohort. When survival is prolonged, age-related CAD risks also increase[31].

Ischemic stroke is a systemic thromboembolic event. Compared to normal subjects, the prevalence of ischemic stroke was reduced in cirrhotic patients[32]. In contrast, hemorrhagic events increased[7,33]. However, in our study, both ischemic and hemorrhagic stroke were significantly increased in nonalcoholic LC subjects. Of note, the prevalence of hemorrhagic stroke in non-alcoholic LC patients was two-fold higher than that in normal subjects (3.4% *vs* 1.7%, *P* < 0.001), which was considerably increased compared to ischemic stroke (10.3% *vs* 8.7%, *P* = 0.005). Older age and higher proportions of concomitant hypertension, DM, and COPD (as a result of smoking) in our nonalcoholic LC cohort might explain this result. These comorbidities have been reported as significant risk factors for all types of stroke[34].

We analyzed the association among comorbidities, complications, and HRs in LC patients. Consistent with previous studies, cirrhosis concomitant with CVD, HF, CKD, or COPD was regarded as a risk factor for mortality in both the Charlson comorbidity index and the Cirrhosis-specific Comorbidity Scoring System (CirCom)[35-37]. In addition, CKD had the highest HR in our study and exhibited increased risk scores in the two prognostic scoring systems. All the complications in LC patients were risk factors in our study. However, CAD, hypertension, and hyperlipidemia were associated with a lower HR and improved survival in this study. Although acute myocardial infarction was found to be a risk factor of mortality in nonalcoholic LC patients[36], the relationship among chronic stable CAD, hypertension, hyperlipidemia, and LC mortality remains unclear. Among these factors, cirrhotic patients with greater than three comorbidities exhibited the worst prognosis with at least a 1.954-fold increased risk of mortality.

Limitations

There were some limitations in our study. First, this is a retrospective cohort study. Although nonalcoholic LC and CAD subjects were retrieved from the Taiwanese

A

Survival rate/yr	0	1	2	3	4	5	6
Non-LC	0.97	0.93	0.91	0.89	0.87	0.85	0.85
LC	0.74	0.67	0.61	0.56	0.53	0.50	0.50

B

Survival rate/yr	0	1	2	3	4	5	6
LC w/ CAD	0.87	0.76	0.70	0.66	0.59	0.52	0.52
LC w/o CAD	0.73	0.66	0.60	0.56	0.52	0.50	0.50

Figure 2 Kaplan-Meier analysis of overall survival. A: Cumulative survival using the Kaplan-Meier method for nonalcoholic LC patients and controls during the follow-up period; B: Cumulative survival using the Kaplan-Meier method for nonalcoholic LC patients with and without CAD during the follow-up period. LC: Liver cirrhosis; CAD: Coronary artery disease.

Table 3 Hazard ratios of mortality in non-alcoholic liver cirrhosis patients with comorbidities using Cox regression model

Variable	Unadjusted hazard ratio			Adjusted hazard ratio[1]		
	Risk ratio	95%CI	*P* value	Risk ratio	95%CI	*P* value
Sex						
Male	1.20	1.07-1.35	0.002	1.34	1.19-1.51	< 0.001
Female (reference)						
Age (yr)						
40-49 (reference)						
50-59	1.04	0.85-1.27	0.688	1.08	0.89-1.32	0.446
60-69	1.29	1.06-1.57	0.013	1.40	1.14-1.72	< 0.001
> 69	2.51	2.10-3.00	< 0.001	2.73	2.25-3.30	< 0.001
Coronary artery disease						
No (reference)						
Yes	0.71	0.55-0.93	0.012	0.56	0.43-0.74	< 0.001
Cerebrovascular disease						
No (reference)						
Yes	1.25	1.08-1.45	0.004	1.08	0.93-1.27	0.323
Hypertension						
No (reference)						
Yes	0.98	0.88-1.09	0.709	0.75	0.66-0.85	< 0.001
Heart failure						
No (reference)						
Yes	1.50	1.26-1.78	< 0.001	1.23	1.02-1.48	0.027
Diabetes mellitus						
No (reference)						
Yes	1.03	0.92-1.16	0.609	1.12	0.99-1.26	0.082
Chronic kidney disease						
No (reference)						
Yes	1.89	1.67-2.13	< 0.001	1.76	1.55-2.00	< 0.001
Hyperlipidemia						
No (reference)						
Yes	0.51	0.44-0.60	< 0.001	0.52	0.44-0.62	< 0.001
Chronic obstructive pulmonary disease						
No (reference)						
Yes	1.30	1.13-1.48	< 0.001	0.99	0.858-1.141	0.888

[1]Adjustments were made for sex, age, coronary heart disease, cerebrovascular disease, hypertension, heart failure, diabetes mellitus, chronic kidney disease, hyperlipidemia, and chronic obstructive pulmonary disease.

NHIRD database according to ICD-9 codes, data were lacking regarding the severity of cirrhosis (*e.g.,* Child-Turcotte-Pugh scores). In this study, we excluded patients who had less than three outpatient visits with the same diagnosis. Furthermore, we analyzed the complications of cirrhosis to evaluate the severity.

Second, the severity of concomitant comorbidities, such as hypertension, DM, and hyperlipidemia, cannot be evaluated. In addition, other known risk factors, such as smoking status and family history of CAD, were not recorded in the database. Third, the severity of CAD was not recorded in the database. A prospective case-

controlled study revealed an increased prevalence of nonobstructive CAD (*i.e.,* narrowing < 50%) in cirrhotic patients compared to normal controls using computerized coronary angiography. This feature may contribute to the increased survival rates for LC patients with CAD. The severity of CAD should be further evaluated in future studies[17]. Finally, the different follow-up periods for each patient may impact the HRs of mortality in nonalcoholic LC patients.

In conclusion, CAD was less prevalent in nonalcoholic LC patients. Among cirrhotic patients, an increased number of concomitant comorbidities was found in cirrhotic patients with CAD. However, improved survival was found in nonalcoholic LC patients with CAD when compared to those without CAD.

ARTICLE HIGHLIGHTS

Research background

The risk of mortality in nonalcoholic liver cirrhosis (LC) patients with coronary artery disease (CAD) is unclear. Previous case-control studies demonstrated conflicting results potentially due to the different etiologies of LC. LC patients with alcoholic and nonalcoholic fatty liver disease-related metabolic disorders exhibit an increased risk of CAD. In contrast, hemostatic defects that occur in LC, such as thrombocytopenia, coagulopathy, and low blood pressure, are not considered as potential protective factors of cirrhosis against atherosclerotic events.

Research motivation

The results of CAD risk in LC patients are controversial. In contrast to the present study, previous works using alcoholic LC-based cohorts may have been confounded by the increased risk of metabolic syndrome in heavy drinkers. To the best of our knowledge, the risk of CAD in nonalcoholic cirrhotic patients is not well established.

Research objectives

The aim of the study was to elucidate the prevalence and risk of mortality in nonalcoholic cirrhotic patients. The comorbidities and LC-related complications were also important prognostic factors among cirrhotic patients. The result of this study can provide a better understanding of CAD risk in LC patients.

Research methods

We collected 10142 LC patients diagnosed from 2004 to 2011 using the Taiwanese National Health Insurance research database. After exclusion of subjects who were treated before the end of 2005, had alcoholic or biliary cirrhosis, had LC occurring after CAD, and were younger than 40 years old, a total of 3409 LC patients were enrolled in the study. The comorbidities and complications of LC were collected. The first-time diagnosis of CAD was identified as the primary end point, and the death of the subject served as the secondary end point. All-cause mortality was analyzed in both the study and control cohorts. A Cox proportional hazards model was developed to calculate the overall mortality hazard ratios of all comorbidities and complications in cirrhotic patients. The six-year cumulative survival and survival curve were calculated using the Cox regression method and the Kaplan-Meier method.

Research results

CAD was less prevalent in nonalcoholic LC patients than in controls. Nonalcoholic LC patients with CAD were associated with a reduced risk of mortality. The six-year survival rates were increased in patients with CAD compared to patients without CAD in the nonalcoholic LC cohort. As this is a retrospective cohort study, further prospective studies are needed to confirm this finding.

Research conclusions

In this study, we demonstrate that CAD is less prevalent and associated with a reduced risk for mortality in nonalcoholic LC patients. This result confirms previous studies regarding the lower risk for atherosclerotic events (*i.e.* ischemic stroke and coronary artery disease) in alcohol-related and nonalcohol-related LC cohorts. Although viral hepatitis can cause endothelial dysfunction, which correlates with atherosclerotic disease progression, we propose that LC has a more powerful protective effect against atherosclerotic events based on the favorable cardiovascular risk profiles, such as thrombocytopenia, coagulopathy, and low blood pressure. The strengths of the study include the large sample size cohort and risk adjustments for comorbidities and complications. Finally, we conclude that nonalcoholic LC patients with CAD exhibit a favorable outcome.

Research perspectives

Future prospective research should focus on the advantages and disadvantages of antiplatelet therapy in the prevention of CAD in nonalcoholic LC patients. Additionally, it is advised that future studies should compare alcoholic and nonalcoholic LC cohorts and evaluate the effect of viral treatment.

REFERENCES

1   **Lozano R**, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn SY, Alvarado M, Anderson HR, Anderson LM, Andrews KG, Atkinson C, Baddour LM, Barker-Collo S, Bartels DH, Bell ML, Benjamin EJ, Bennett D, Bhalla K, Bikbov B, Bin Abdulhak A, Birbeck G, Blyth F, Bolliger I, Boufous S, Bucello C, Burch M, Burney P, Carapetis J, Chen H, Chou D, Chugh SS, Coffeng LE, Colan SD, Colquhoun S, Colson KE, Condon J, Connor MD, Cooper LT, Corriere M, Cortinovis M, de Vaccaro KC, Couser W, Cowie BC, Criqui MH, Cross M, Dabhadkar KC, Dahodwala N, De Leo D, Degenhardt L, Delossantos A, Denenberg J, Des Jarlais DC, Dharmaratne SD, Dorsey ER, Driscoll T, Duber H, Ebel B, Erwin PJ, Espindola P, Ezzati M, Feigin V, Flaxman AD, Forouzanfar MH, Fowkes FG, Franklin R, Fransen M, Freeman MK, Gabriel SE, Gakidou E, Gaspari F, Gillum RF, Gonzalez-Medina D, Halasa YA, Haring D, Harrison JE, Havmoeller R, Hay RJ, Hoen B, Hotez PJ, Hoy D, Jacobsen KH, James SL, Jasrasaria R, Jayaraman S, Johns N, Karthikeyan G, Kassebaum N, Keren A, Khoo JP, Knowlton LM, Kobusingye O, Koranteng A, Krishnamurthi R, Lipnick M, Lipshultz SE, Ohno SL, Mabweijano J, MacIntyre MF, Mallinger L, March L, Marks GB, Marks R, Matsumori A, Matzopoulos R, Mayosi BM, McAnulty JH, McDermott MM, McGrath J, Mensah GA, Merriman TR, Michaud C, Miller M, Miller TR, Mock C, Mocumbi AO, Mokdad AA, Moran A, Mulholland K, Nair MN, Naldi L, Narayan KM, Nasseri K, Norman P, O'Donnell M, Omer SB, Ortblad K, Osborne R, Ozgediz D, Pahari B, Pandian JD, Rivero AP, Padilla RP, Perez-Ruiz F, Perico N, Phillips D, Pierce K, Pope CA 3rd, Porrini E, Pourmalek F, Raju M, Ranganathan D, Rehm JT, Rein DB, Remuzzi G, Rivara FP, Roberts T, De León FR, Rosenfeld LC, Rushton L, Sacco RL, Salomon JA, Sampson U, Sanman E, Schwebel DC, Segui-Gomez M, Shepard DS, Singh D, Singleton J, Sliwa K, Smith E, Steer A, Taylor JA, Thomas B, Tleyjeh IM, Towbin JA, Truelsen T, Undurraga EA, Venketasubramanian N, Vijayakumar L, Vos T, Wagner GR, Wang M, Wang W, Watt K, Weinstock MA, Weintraub R, Wilkinson JD, Woolf AD, Wulf S, Yeh PH, Yip P, Zabetian A, Zheng ZJ, Lopez AD, Murray CJ, AlMazroa MA, Memish ZA. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012; **380**: 2095-2128 [PMID: 23245604 DOI: 10.1016/S0140-6736(12)61728-0]

2   **Department of Health EY**, ROC. Statistics of Causes of Death. 2011

3   **Tsochatzis EA**, Bosch J, Burroughs AK. Liver cirrhosis. *Lancet* 2014; **383**: 1749-1761 [PMID: 24480518 DOI: 10.1016/S0140-6736(14)60121-5]

4   **Amin J**, Law MG, Bartlett M, Kaldor JM, Dore GJ. Causes of death after diagnosis of hepatitis B or hepatitis C infection: a large community-based linkage study. *Lancet* 2006; **368**: 938-945 [PMID: 16962883 DOI: 10.1016/S0140-6736(06)69374-4]

5   **Sun LM**, Lin MC, Lin CL, Liang JA, Jeng LB, Kao CH, Lu

CY. Nonalcoholic Cirrhosis Increased Risk of Digestive Tract Malignancies: A Population-Based Cohort Study. *Medicine* (Baltimore) 2015; **94**: e2080 [PMID: 26656334 DOI: 10.1097/MD.0000000000002080]

6   **Jepsen P**, Lash TL, Vilstrup H. The clinical course of alcoholic cirrhosis: development of comorbid diseases. A Danish nationwide cohort study. *Liver Int* 2016; **36**: 1696-1703 [PMID: 27124269 DOI: 10.1111/liv.13151]

7   **Parikh NS**, Navi BB, Kumar S, Kamel H. Association between Liver Disease and Intracranial Hemorrhage. *J Stroke Cerebrovasc Dis* 2016; **25**: 543-548 [PMID: 26679070 DOI: 10.1016/j.jstrokecerebrovasdis.2015.11.005]

8   **Berzigotti A**, Bonfiglioli A, Muscari A, Bianchi G, Libassi S, Bernardi M, Zoli M. Reduced prevalence of ischemic events and abnormal supraortic flow patterns in patients with liver cirrhosis. *Liver Int* 2005; **25**: 331-336 [PMID: 15780058 DOI: 10.1111/j.1478-3231.2005.01002.x]

9   **Tiukinhoy-Laing SD**, Rossi JS, Bayram M, De Luca L, Gafoor S, Blei A, Flamm S, Davidson CJ, Gheorghiade M. Cardiac hemodynamic and coronary angiographic characteristics of patients being evaluated for liver transplantation. *Am J Cardiol* 2006; **98**: 178-181 [PMID: 16828588 DOI: 10.1016/j.amjcard.2006.01.089]

10 **HOWELL WL**, MANION WC. The low incidence of myocardial infarction in patients with portal cirrhosis of the liver: A review of 639 cases of cirrhosis of the liver from 17,731 autopsies. *Am Heart J* 1960; **60**: 341-344 [PMID: 14403495 DOI: 10.1016/0002-8703(60)90192-7]

11 **Carey WD**, Dumot JA, Pimentel RR, Barnes DS, Hobbs RE, Henderson JM, Vogt DP, Mayes JT, Westveer MK, Easley KA. The prevalence of coronary artery disease in liver transplant candidates over age 50. *Transplantation* 1995; **59**: 859-864 [PMID: 7701580 DOI: 10.1097/00007890-199503270-00010]

12 **Mukamal KJ**, Rimm EB. Alcohol's effects on the risk for coronary heart disease. *Alcohol Res Health* 2001; **25**: 255-261 [PMID: 11910702]

13 **Kalaitzakis E**, Björnsson E. Coronary artery disease in liver cirrhosis: does the aetiology of liver disease matter? *J Hepatol* 2009; **51**: 962-963; author reply 963-964 [PMID: 19692136 DOI: 10.1016/j.jhep.2009.06.012]

14 **Marui A**, Kimura T, Tanaka S, Miwa S, Yamazaki K, Minakata K, Nakata T, Ikeda T, Furukawa Y, Kita T, Sakata R; CREDO-Kyoto Investigators. Coronary revascularization in patients with liver cirrhosis. *Ann Thorac Surg* 2011; **91**: 1393-1399 [PMID: 21396626 DOI: 10.1016/j.athoracsur.2011.01.022]

15 **Raval Z**, Harinstein ME, Skaro AI, Erdogan A, DeWolf AM, Shah SJ, Fix OK, Kay N, Abecassis MI, Gheorghiade M, Flaherty JD. Cardiovascular risk assessment of the liver transplant candidate. *J Am Coll Cardiol* 2011; **58**: 223-231 [PMID: 21737011 DOI: 10.1016/j.jacc.2011.03.026]

16 **Gologorsky E**, Pretto EA Jr, Fukazawa K. Coronary artery disease and its risk factors in patients presenting for liver transplantation. *J Clin Anesth* 2013; **25**: 618-623 [PMID: 23994032 DOI: 10.1016/j.jclinane.2013.06.001]

17 **An J**, Shim JH, Kim SO, Lee D, Kim KM, Lim YS, Lee HC, Chung YH, Lee YS. Prevalence and prediction of coronary artery disease in patients with liver cirrhosis: a registry-based matched case-control study. *Circulation* 2014; **130**: 1353-1362 [PMID: 25095888 DOI: 10.1161/CIRCULATIONAHA.114.009278]

18 **Tripodi A**, Mannucci PM. The coagulopathy of chronic liver disease. *N Engl J Med* 2011; **365**: 147-156 [PMID: 21751907 DOI: 10.1056/NEJMra1011170]

19 **D'Arienzo A**, Manguso F, Scaglione G, Vicinanza G, Bennato R, Mazzacca G. Prognostic value of progressive decrease in serum cholesterol in predicting survival in Child-Pugh C viral cirrhosis. *Scand J Gastroenterol* 1998; **33**: 1213-1218 [PMID: 9867102 DOI: 10.1080/00365529850172593]

20 **Lisman T**, Caldwell SH, Burroughs AK, Northup PG, Senzolo M, Stravitz RT, Tripodi A, Trotter JF, Valla DC, Porte RJ; Coagulation in Liver Disease Study Group. Hemostasis and thrombosis in patients with liver disease: the ups and downs. *J Hepatol* 2010; **53**: 362-371 [PMID: 20546962 DOI: 10.1016/j.jhep.2010.01.042]

21 **Kadayifci A**, Tan V, Ursell PC, Merriman RB, Bass NM. Clinical and pathologic risk factors for atherosclerosis in cirrhosis: a comparison between NASH-related cirrhosis and cirrhosis due to other aetiologies. *J Hepatol* 2008; **49**: 595-599 [PMID: 18662837 DOI: 10.1016/j.jhep.2008.05.024]

22 **Kalaitzakis E**, Rosengren A, Skommevik T, Björnsson E. Coronary artery disease in patients with liver cirrhosis. *Dig Dis Sci* 2010; **55**: 467-475 [PMID: 19242795 DOI: 10.1007/s10620-009-0738-z]

23 **Morgan TR**, Ghany MG, Kim HY, Snow KK, Shiffman ML, De Santo JL, Lee WM, Di Bisceglie AM, Bonkovsky HL, Dienstag JL, Morishima C, Lindsay KL, Lok AS; HALT-C Trial Group. Outcome of sustained virological responders with histologically advanced chronic hepatitis C. *Hepatology* 2010; **52**: 833-844 [PMID: 20564351 DOI: 10.1002/hep.23744]

24 **European Association For The Study Of The Liver.**. EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. *J Hepatol* 2012; **57**: 167-185 [PMID: 22436845 DOI: 10.1016/j.jhep.2012.02.010]

25 **Mokdad AA**, Lopez AD, Shahraz S, Lozano R, Mokdad AH, Stanaway J, Murray CJ, Naghavi M. Liver cirrhosis mortality in 187 countries between 1980 and 2010: a systematic analysis. *BMC Med* 2014; **12**: 145 [PMID: 25242656 DOI: 10.1186/s12916-014-0145-y]

26 **Kane WC**, Aronson SM. Cerebrovascular disease in an autopsy population. 4. Reduced frequency of stroke in patients with liver cirrhosis. *Trans Am Neurol Assoc* 1971; **96**: 259-260 [PMID: 5159099]

27 **Patel S**, Kiefer TL, Ahmed A, Ali ZA, Tremmel JA, Lee DP, Yeung AC, Fearon WF. Comparison of the frequency of coronary artery disease in alcohol-related versus non-alcohol-related endstage liver disease. *Am J Cardiol* 2011; **108**: 1552-1555 [PMID: 21890080 DOI: 10.1016/j.amjcard.2011.07.013]

28 **Ciccone MM**, Principi M, Ierardi E, Di Leo A, Ricci G, Carbonara S, Gesualdo M, Devito F, Zito A, Cortese F, Scicchitano P. Inflammatory bowel disease, liver diseases and endothelial function: is there a linkage? *J Cardiovasc Med* (Hagerstown) 2015; **16**: 11-21 [PMID: 25427048 DOI: 10.2459/JCM.0000000000000149]

29 **Butt AA**, Xiaoqiang W, Budoff M, Leaf D, Kuller LH, Justice AC. Hepatitis C virus infection and the risk of coronary disease. *Clin Infect Dis* 2009; **49**: 225-232 [PMID: 19508169 DOI: 10.1086/599371]

30 **Roed T**, Lebech AM, Kjaer A, Weis N. Hepatitis C virus infection and risk of coronary artery disease: a systematic review of the literature. *Clin Physiol Funct Imaging* 2012; **32**: 421-430 [PMID: 23031062 DOI: 10.1111/j.1475-097X.2012.01152.x]

31 **Savji N**, Rockman CB, Skolnick AH, Guo Y, Adelman MA, Riles T, Berger JS. Association between advanced age and vascular disease in different arterial territories: a population database of over 3.6 million subjects. *J Am Coll Cardiol* 2013; **61**: 1736-1743 [PMID: 23500290 DOI: 10.1016/j.jacc.2013.01.054]

32 **Chen YH**, Chen KY, Lin HC. Non-alcoholic cirrhosis and the risk of stroke: a 5-year follow-up study. *Liver Int* 2011; **31**: 354-360 [PMID: 20860634 DOI: 10.1111/j.1478-3231.2010.02350.x]

33 **Lai CH**, Cheng PY, Chen YY. Liver cirrhosis and risk of intracerebral hemorrhage: a 9-year follow-up study. *Stroke* 2011; **42**: 2615-2617 [PMID: 21757678 DOI: 10.1161/STROKEAHA.111.617076]

34 **O'Donnell MJ**, Xavier D, Liu L, Zhang H, Chin SL, Rao-Melacini P, Rangarajan S, Islam S, Pais P, McQueen MJ, Mondo C, Damasceno A, Lopez-Jaramillo P, Hankey GJ, Dans AL, Yusoff K, Truelsen T, Diener HC, Sacco RL, Ryglewicz D, Czlonkowska A, Weimar C, Wang X, Yusuf S; INTERSTROKE investigators. Risk factors for ischaemic and intracerebral haemorrhagic stroke in 22 countries (the INTERSTROKE study): a case-control study. *Lancet* 2010; **376**: 112-123 [PMID: 20561675 DOI: 10.1016/S0140-6736(10)60834-3]

35 **Charlson ME**, Pompei P, Ales KL, MacKenzie CR. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. *J Chronic Dis* 1987; **40**: 373-383 [PMID: 3558716 DOI: 10.1016/0021-9681(87)90171-8]

36   **Jepsen P**, Vilstrup H, Andersen PK, Lash TL, Sørensen HT. Comorbidity and survival of Danish cirrhosis patients: a nationwide population-based cohort study. *Hepatology* 2008; **48**: 214-220 [PMID: 18537190 DOI: 10.1002/hep.22341]

37   **Jepsen P**, Vilstrup H, Lash TL. Development and validation of a comorbidity scoring system for patients with cirrhosis. *Gastroenterology* 2014; **146**: 147-156; quiz e15-e16 [PMID: 24055278 DOI: 10.1053/j.gastro.2013.09.019]

ORIGINAL ARTICLE

Prospective Study

PillCamColon2 after incomplete colonoscopy - A prospective multicenter study

Peter Baltes, Marc Bota, Jörg Albert, Michael Philipper, Hans-Georg Hörster, Friedrich Hagenmüller, Ingo Steinbrück, Ralf Jakobs, Matthias Bechtler, Dirk Hartmann, Horst Neuhaus, Jean-Pierre Charton, Rupert Mayershofer, Horst Hohn, Thomas Rösch, Stefan Groth, Tanja Nowak, Peter Wohlmuth, Martin Keuchel

Peter Baltes, Marc Bota, Martin Keuchel, Clinic for Internal Medicine, Bethesda Krankenhaus Bergedorf, Hamburg 21029, Germany

Jörg Albert, Department of Internal Medicine I, Klinikum der JW. Goethe Universität, Frankfurt 60590, Germany

Michael Philipper, Gastroenterologische Schwerpunktpraxis, Düsseldorf 40227, Germany

Hans-Georg Hörster, Gastropraxis Rheydt, Mönchengladbach 41236, Germany

Friedrich Hagenmüller, Ingo Steinbrück, 1st Medical Department, Asklepios Klinikum Altona, Hamburg 22763, Germany

Ralf Jakobs, Matthias Bechtler, Medical Clinic C, Klinikum der Stadt Ludwigshafen, Ludwigshafen 67063, Germany

Dirk Hartmann, Clinic for Internal Medicine, Sana Klinikum Lichtenberg, Berlin 10365, Germany

Horst Neuhaus, Jean-Pierre Charton, Clinic for Internal Medicine, Evangelisches Krankenhaus, Düsseldorf 40217, Germany

Rupert Mayershofer, Gastroenterologie am Burgweiher, Bonn 53123, Germany

Horst Hohn, Schwerpunktpraxis Gastroenterologie, Koblenz 56068, Germany

Thomas Rösch, Stefan Groth, Clinic for Interdisciplinary Endoscopy, University Hospital Eppendorf, Hamburg 20251, Germany

Tanja Nowak, CorporateHealth International, Hamburg 20149, Germany

Peter Wohlmuth, Biometry and Data Management, Asklepios Proresearch, Hamburg 20099, Germany

ORCID number: Peter Baltes (0000-0001-6318-6015); Marc Bota (0000-0003-0265-8642); Jörg Albert (0000-0002-3420-7371); Michael Philipper (0000-0002-9557-9662); Hans-Georg Hörster (0000-0003-0563-6596); Friedrich Hagenmüller (0000-0001-7436-8527); Ingo Steinbrück (0000-0002-8861-2903); Ralf Jakobs (0000-0002-1902-5692); Matthias Bechtler (0000-0002-2449-5373); Dirk Hartmann (0000-0002-9783-6431); Horst Neuhaus (0000-0001-6200-0688); Jean-Pierre Charton (0000-0003-4654-3062); Rupert Mayershofer (0000-0001-8310-1381); Horst Hohn (0000-0001-7931-6910); Thomas Rösch (0000-0003-2270-2495); Stefan Groth (0000-0003-2022-8806); Tanja Nowak (0000-0002-2648-087X); Peter Wohlmuth (0000-0002-1636-7865); Martin Keuchel (0000-0001-5288-4993).

CONSORT 2010 statement, and the manuscript was prepared and revised according to the CONSORT 2010 Statement.

Correspondence to: Peter Baltes, MD, Doctor, Senior Consultant, Clinic for Internal Medicine, Bethesda Krankenhaus Bergedorf, Akademisches Lehrkrankenhaus der Universität Hamburg, Glindersweg 80, Hamburg 21029, Germany. baltes@bkb.info
Telephone: +49-40-725541201
Fax: +49-40-725541202

Abstract

AIM

To evaluate the ability of PillCamColon2 to visualize colonic segments missed by incomplete optical colonoscopy (OC) and to assess the diagnostic yield.

METHODS

This prospective multicentre study included 81 patients from nine centres who underwent second-generation colon capsule endoscopy (CCE) following incomplete OC performed by an experienced gastroenterologist (> 1000 colonoscopies). Patients with stenosis were excluded. According to patient preferences, CCE was performed the following day (protocol A) after staying on clear liquids and 0.75 L Moviprep in the morning or within 30 d after new split-dose Moviprep (protocol B). Boosts consisted of 0.75 L and 0.25 L Moviprep, and phospho-soda was given as a rescue if the capsule was not excreted after seven hours.

RESULTS

Seventy-four patients were analysed (51% of them in group A; 49% in group B). Bowel cleansing was adequate in 67% of cases, and CCE could visualize colonic segments missed by incomplete colonoscopy in 90% of patients under protocol A and 97% of patients under protocol B (P = 0.35, n.s.). Significant polyps including adenocarcinoma were detected in 24% of cases. Detection rates for all polyps and significant polyps per patient were similar in both protocols. Polyps were found predominantly in the right colon (86%) in segments that were not reached by OC. Extracolonic findings - such as reflux esophagitis, suspected Barrett esophagus, upper GI-bleeding, gastric polyps, gastric erosions and angiectasia - were detected in eight patients. PillCamColon2 capsule was retained in the ileum of one patient (1.4%) without symptoms and removed during an uneventful resection for unknown Crohn's disease that was diagnosed as the cause of anemia, which was the indication for colonoscopy. CCE was well tolerated. One patient suffered from self-limiting vomiting after consuming the phospho-soda.

CONCLUSION

Second-generation CCE using a low-volume preparation is useful after incomplete OC, and it allows for the detection of additional relevant findings, but cleansing efficiency could be improved.

Key words: Colon capsule endoscopy; PillCamColon2; Incomplete colonoscopy; Low volume prep; Moviprep; Phospho-soda; Cleanliness level; Complementation rate; Polyps

Core tip: Colonoscopy is the gold standard for visualization of the colon, but it may be incomplete, not reaching the cecum. Second-generation colon capsule endoscopy (CCE) with low-volume preparations could complement incomplete colonoscopies in 90% of cases, and it could help to detect additional relevant colonic and extracolonic findings. Protocols with either CCE the day following an incomplete colonoscopy or within 30 d after a new low-volume preparation were both feasible and well tolerated; however, the protocols could be improved with respect to bowel cleanliness and complete colon visualization.

Baltes P, Bota M, Albert J, Philipper M, Hörster HG, Hagenmüller F, Steinbrück I, Jakobs R, Bechtler M, Hartmann D, Neuhaus H, Charton JP, Mayershofer R, Hohn H, Rösch T, Groth S, Nowak T, Wohlmuth P, Keuchel M. PillCamColon2 after incomplete colonoscopy - A prospective multicenter study. *World J Gastroenterol* 2018; 24(31): 3556-3566 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i31/3556.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i31.3556

INTRODUCTION

Flexible optical colonoscopy (OC) is the gold standard to detect and treat colorectal diseases and proved to be safe and effective in colorectal cancer screening (CRC)[1]. However, OC may be incomplete for various reasons, with completion rates between 90% and 98%[2-5] for screening colonoscopy and 81%-94% in mixed series[6-9]. Risk factors for incomplete colonoscopy

are female gender, elder patients, previous pelvic or abdominal surgery, diverticulosis, redundant colon, stenosing tumors, poor preparation, inflammatory bowel disease; in contrast sedation, male gender and higher body mass index were reported as protective factors[4,6,7,10,11]. Of the patients with incomplete OC, 4.3% had advanced neoplasia in the right colon[5]. A five-year follow-up study showed a higher risk for colorectal cancer in patients with failed colonoscopy[12], demonstrating the need for additional investigations in these patients. PillCamColon has been cleared by the US Food and Drug Administration (FDA) for detection of colon polyps in patients after an incomplete optical colonoscopy with adequate preparation. Later the indication had been expanded to the detection of colon polyps in patients with evidence of lower gastrointestinal origin and major risks for colonoscopy or moderate sedation.

First-generation PillCamColon1 with a constant rate of two frames per second (fps) had limited sensitivity compared to a colonoscopy[13-15]. Technical improvements of the second-generation PillCamColon2 that was used in the present study included adaptive frame rates (4 fps and 35 fps) and an increased viewing angle. It also included automatic small bowel detection with consecutive timing of boosters to shorten transit times and to improve cleansing levels[16], which was shown to have a significantly higher diagnostic yield for polyps[17]. A decisive factor for CCE accuracy is bowel cleansing; recent data have shown that low-volume Polyethylene glycol (PEG)-based protocols may be feasible instead of high-volume preparations[18,19].

Our study aimed to assess the impact of second-generation CCE after incomplete colonoscopies by analyzing the complementation rates and incremental diagnostic yields with two different low-volume cleansing protocols. Patients were offered to either stay on clear liquids with a CCE the next day or to start a new bowel preparation procedure within 30 d.

MATERIALS AND METHODS

Study design and population

This prospective, multicentre study including nine centres (tertiary care hospitals or private endoscopy offices) in Germany. It was approved by the ethics committee of Hamburg Chamber of Physicians (PV3467, 20.05.2010). The www.clinicaltrials.gov identifier is NCT01480635.

All patients included in the study were 18 years or older who had incomplete colonoscopies that were performed by an experienced endoscopist (> 1000 colonoscopies performed). Incomplete OC was defined as a failure to reach the cecum or ileo-cecal anastomosis due to looping, bowel angulation, adhesions, and intolerance of sedation or inflammation. The presumed area of the colon reached by OC was documented, as were reasons for termination, detection of polyps and

tumours, other findings and adverse events. Patients with stenosis, inadequate preparation, or exchange of endoscope were excluded. Other exclusion criteria for CCE have been described previously[18]. Written informed consent was obtained from all patients.

Study protocol

After an incomplete colonoscopy, patients were rescheduled for the following day (protocol A) or they were given a separate appointment within 30 d (protocol B), according to their preference. CCE was performed with PillCamColon2 (Given Imaging, Yoqneam, Israel) after a low-volume cleansing regimen with PEG and ascorbic acid (Moviprep, Norgine, Marburg/Lahn, Germany). Following capsule ingestion, the boosts consisted of 0.75 L after small bowel detection and 0.25 L Moviprep if the capsule had not been excreted five hours after ingestion. 30 mL sodium picosulfate (NaP; Fleet, Recordati, Ulm, Germany) was used as an additional boost if the capsule was not excreted seven hours after ingestion. In protocol A, patients stayed on clear liquids after the colonoscopy, and they received an additional cleansing of 0.75 L Moviprep the next morning (at the latest, one hour before CCE). In protocol B, patients were allowed to eat after the OC. A new bowel cleansing procedure was performed within 30 d: split-dose 1 L of Moviprep was consumed in the evening and 1 L was consumed in the morning, each followed by 1 L of water. Boosts with Moviprep or NaP were identical in both protocols (Table 1).

CCE studies were read with Rapid7 or Rapid8 software in each centre by an experienced endoscopist with additional experience in capsule endoscopy (> 1000 colonoscopies, > 100 small bowel capsule endoscopies (SBCE) and > 25 CCEs performed). All readers had completed a dedicated two-day CCE evaluation course. Polyp size was estimated with the integrated software tool.

For both examination modalities, cleansing levels were documented, as described previously[15], using four grades: excellent, good (adequate), fair and poor (inadequate). Each colon segment [that is, cecum, ascending colon (AC), transverse colon (TC), left colon (LC) and rectum, see Figure 1] and overall cleansing status were evaluated.

For CCE, visualization of colonic segments, complementation of previous colonoscopy, completeness of CCE, and adverse events were recorded. Complete CCE was defined as excretion of capsule during recording time or by identification of the haemorrhoidal plexus. Detection of polyps, significant polyps, tumours or other relevant findings were documented for segments reached and not reached by the previous standard colonoscopy. According to previous studies, significant polyps were defined by size ($\geq$ 6 mm) or number ($\geq$ 3)[19-21]. Other findings were considered important if they explained the indication for the colonoscopy or if they had further diagnostic or therapeutic implications.

Table 1 Study protocols A and B for colon capsule endoscopy after incomplete optical colonoscopy

Time	Procedure	
Evening and morning before colonoscopy	Standard bowel prep for colonoscopy	
	Incomplete colonoscopy (OC)	
After incomplete colonoscopy	Patient´s choice of protocol A or B for colon capsule endoscopy (CCE)	
	Protocol A (CCE next day)	**Protocol B** (CCE within 30 d)
After incomplete colonoscopy	Patient stays on clear liquids	Patient can eat
2 d before CCE		Low residue diet
Day before CCE	NA	Clear liquids only
Evening before CCE		1 L Moviprep + 1 L water
Morning of CCE	0.75 L Moviprep + water	1 l Moviprep + 1 L water
	Colon capsule ingestion	
Small bowel detection	0.75 L Moviprep + water (1st boost)	
5 h after ingestion	0.5 L Moviprep + water (2nd boost)[1]	
7 h after ingestion	30 mL NaP + water (´Rescue boost)[1]	
	Bisacodyl supp[1]	
11 h after ingestion	Removal of equipment[1]	

[1]If capsule is not excreted earlier. OC: Optical colonoscopy.

Follow-up took place via a telephone call one week after the procedure. During the call, adverse events were documented, and capsule excretion was confirmed. Any adverse events were recorded, as well as the time of capsule excretion.

Study endpoints

The primary outcome parameter was complementation rate of CCE in patients with incomplete OC, which was defined by visualization of colonic segments not reached by OC. The secondary outcome parameters were as follows: additional (incremental) diagnostic yield of CCE compared to incomplete OC (all polyps, significant polyps defined by size or number and other significant findings) in segments not reached by OC; CCE findings in the upper gastrointestinal (GI) tract and small bowel; rate of complete CCE, as defined above; cleansing level of the colon (overall and segments) following the low-volume protocol for CCE; visualization of the Z-Line; and adverse events (number, type and severity).

Statistical analysis

Continuous variables were reported as mean and standard deviation, and categorical variables were reported as percentage. The null hypothesis (H0) for the primary endpoint (complementation of incomplete colonoscopy) was constructed using data from a former study that used PillCamColon1 and that showed a complementation rate of 50%[22]. Accordingly, H0 was set to $\mu = 0.5$ with an expectation for complementation of OC by PillCamColon2 of 0.67. Using the Fisher exact test with a power of 80% (which is equal to $1-\beta$), 74 patients had to be recruited. Categorical values were compared using a chi-squared test (χ^2), while continuous values with a normal distribution were compared by a Student´s *t*-test. *P* values < 0.05 were considered significant. An intention-to-treat (ITT) analysis was performed for complementation rates, cleansing levels, detection of significant polyps and safety. Statistical review was performed by one of the authors (Peter Wohlmuth).

RESULTS

Demographics

Eighty-one consecutive patients were enrolled from nine participating centres between 2010 and 2013. Seven patients (four in the protocol A group and three in the protocol B group) had to be excluded due to technical failure ($n = 1$), protocol noncompliance as a result of incorrect timing of CCE ($n = 4$), exchange of colonoscope ($n = 1$) or early removal of recorder by the patient ($n = 1$) (Figure 2). In total, data of 74 patients were analysed per protocol. Demographics are shown in Table 2.

Reasons for referral to colonoscopy were CRC screening (22%), anemia (15%), hematochezia (15%), irregular stool (12%), abdominal pain (12%), B symptoms (7%), colitis (5%) and other reasons (12%). Thirty-six patients (48.6%) had prior abdominal surgery, while 14 patients (19%) had more than one surgical intervention. Most common surgeries were appendectomies (23%) and hysterectomies (19%). Only three patients had colonic surgery (4%), one patient had an ileocecal and one patient had a Billroth Ⅱ anastomosis.

Standard OC

An experienced endoscopist (> 1000 colonoscopies performed) performed OC with a standard colonoscope. The mean duration of the procedure was 45 ± 17 min (range: 15-101 min). Unfavourable anatomy was the reason for termination in 92% of procedures. OC reached the sigmoid colon in 27% of cases, the descending colon (DC) in 4% of cases, the splenic flexure in 12% of cases, the TC in 14% of cases, the hepatic flexure in 35% of cases and the AC in 8% of cases. Adequate cleansing was achieved in 76% of procedures. In 12 of 74 patients (16%), polyps were detected, with a mean size of 6 ± 4.2 mm. Six

Figure 1 Landmarks at colon capsule endoscopy. A: Terminal ileum; B: IC valve; C: Appendix; D: Hemorrhoidal plexus, hepatic flexure - CCE image (E) with corresponding localization trace (G), white circle showing actual capsule position, green colon already displayed, grey colon yet to be analyzed, orange small bowel, blue stomach, outer pictogram the position to the colonic segment as manually defined by setting the landmarks; F and H: CCE image and localization trace of splenic flexure in another patient. CCE: Colon capsule endoscopy.

patients had more than one polyp (range: 2-5 polyps). Diverticula were found in 35 patients. Other findings were diverticulitis, erosions, angiectasias and erythema.

Colon capsule endoscopy
Primary endpoint: complementation of incomplete colonoscopy: Incomplete colonoscopy could

be complemented by CCE in 69 of 74 patients (93%; see Table 2). Complete CCE was achieved in 48 of 74 patients (65%). In four additional patients, the capsule reached the rectum but did not visualize the haemorrhoidal plexus (5%).

Complementation of OC could be achieved by CCE with protocol A in 89.5% of procedures and with protocol

Table 2 Demographics, reasons for termination of optical colonoscopy, and results of colon capsule endoscopy for protocol A and B *n* (%)

	Protocol A (CCE next day)	Protocol B (CCE within 30 d)	Significance
Demographics			
Patients	38 (51.4)	36 (48.6)	
Female	20/38 (52.6)	24/36 (66.7)	
Age, mean ± SD	68.0 ± 12.8 yr	63.9 ± 13.0 yr	
Body mass index	26.0 ± 3.9	26.5 ± 4.9	
Reasons for termination of colonoscopy			
Looping of colon	23/38 (60.5)	23/36 (63.9)	
Angulation of colon	6/38 (15.8)	5/36 (13.9)	
Susp. adhesions	5/38 (13.2)	6/36 (16.7)	
Risk of perforation	2/38 (5.3)	0	$P = 0.710$ (NS); χ^2 test
Sedation problems	2/38 (5.3)	2/36 (5.6)	
Results of CCE			
Complete CCE	24/38 (63.3)	24/36 (66.7)	$P = 0.560$ (NS); χ^2 test
Complementation of colonoscopy	34/38 (89.5)	35/36 (97.2)	$P = 0.350$ (NS); χ^2 test
Adequate cleansing	25/36 (69.4)	23/36 (63.9)	$P = 0.820$ (NS); χ^2 test
Patients with significant colon polyps	10/38 (26.3)	11/36 (30.6)	$P = 0.500$ (NS); χ^2 test
Patients with other colon findings	0	Angiectasia ($n = 3$)	$P = 0.045$; χ^2 test
		Diverticulitis ($n = 1$)	
Patients with small bowel findings	Angiectasia ($n = 1$)	0	$P = 0.174$ (NS); χ^2 test
	Crohn's disease ($n = 1$)		
Patients with upper GI findings	Reflux esophagitis ($n = 1$)	Reflux-esophagitis ($n = 1$)	$P = 0.949$ (NS); χ^2 test
	Upper GI-bleeding ($n = 1$)	Susp. Barrett esophagus ($n = 1$)	
	Gastric polyps ($n = 1$)	Gastric erosions ($n = 1$)	

CCE: Colon capsule endoscopy.

Figure 2 Flow chart protocol A (colon capsule endoscopy the day after colonoscopy) and protocol B (colon capsule endoscopy within 30 d after incomplete colonoscopy).

B in 97.2% of procedures ($P = 0.35$, not significant; Table 2). There were no differences between the rates of complementation using CCE for protocol A or B and the diagnostic yield. In the patients with incomplete visualization of the missing segments ($n = 5$), the capsule was able to display the hepatic flexure in two patients. One patient had a capsule retention in the small bowel because of unknown stenosing Crohn's disease. In one patient, the capsule did not reach the colon during recording time, and in one patient, visualization of the colon was incomplete due to recording gaps. The capsule was excreted within seven hours of ingestion and before the need for an additional NaP booster in 17 of 38 patients (44.7%) following protocol A, and in 12 of 36 patients (33.3%; $P = 0.25$, not significant) in protocol B.

Secondary endpoints: CCE was performed on the

day after colonoscopy in 38 patients (protocol A; 51%) or within 30 d in 36 patients (protocol B; 49%). Overall, cleansing was adequate in 48 of 72 (67%), and cleansing was adequate in the cecum in 58% of cases, in the AC in 65% of cases, in the TC in 77% of cases, in the LC in 70% of cases and in the rectum in 63% of cases (Figure 3); there were no differences between the protocols. Two capsules did not allow for visualization of the colon. Poor cleansing was rare (4 of 72 patients; 5.6%).

CCE detected 76 polyps in 35 of 74 patients (47%; Figure 4). Twenty-one patients (28%) had significant polyps (Table 3), and 14 patients had an insignificant number of polyps. In 9 of 21 patients, polyp size was ≥ 6 mm; in 3 patients, the number of polyps was ≥ 3; and in 9 patients, both parameters were positive. A total of 59 polyps (mean size 8 ± 4.5 mm) were detected in the 21

A

Cleansing levels
Protocol a (CCE next day)

B

Cleansing levels
Protocol b (CCE within 30 d)

C

Cleansing levels
Incomplete colonoscopy

Figure 3 Cleansing levels for the colon segments: cecum, ascending colon, transverse colon, left colon, rectum and overall classification for all segments. Absolute number of patients for each level (poor, fair, good, excellent) are shown A for CCE with protocol a (next day), B for CCE with protocol b (within 30 d), and C for incomplete colonoscopy. AC: Ascending colon; CCE: Colon capsule endoscopy; LC: Left colon; TC: Transverse colon.

patients with significant polyps. Significant polyps were predominantly found in the ascending colon in segments that had not been reached by OC (86%). Incremental diagnostic yield of CCE compared to previous incomplete OC per patient for detecting significant polyps was 24%, and there were no differences between the two protocols. A cecal polyp of 26 mm turned out to be an adenocarcinoma, and hemicolectomy was performed in this patient.

Z-line was visualized in 45 of 74 patients (60.8%). CCE findings in the upper GI tract were reflux esophagitis (n = 2), suspected Barrett´s esophagus (n = 1), hemorrhagic gastropathy (n = 1), gastric polyps (n = 1; consecutive gastroscopy revealed foveolar hyperplasia in previously undiagnosed atrophic gastritis with vitamin B12 deficiency) and upper GI bleeding (n = 1).

In the small bowel, angiectasia and previously unknown Crohn's disease was detected in one patient each. In colon segments missed by incomplete colonoscopy, angiectasias were detected in three patients and diverticulitis was detected in one patient (all patients were part of protocol B).

ITT analysis of all 81 patients found a complementation rate of 89%, adequate cleansing in 65%, significant polyps in 26% of patients, and no additional adverse events. The low complementation rate that was observed in this cohort is consistent with the exclusion criteria [technical problems, n = 1, and noncompliance of study protocol, n = 6 (for example, early removal of recorder and incorrect CCE timing)].

In one patient (protocol A), the capsule was retained in the small bowel without symptoms. Surgery was indicated following a new diagnosis of stenosing and fistulating Crohn's disease with iron deficiency anemia (indication for

colonoscopy). During an uneventful surgery, the capsule was retrieved. Another patient (protocol B) complained of self-limiting nausea and vomiting after the NaP boost.

DISCUSSION

In this prospective, multicentre study, second-generation CCE allowed for the visualization of colon segments that were not reached by a previously incomplete colonoscopy in 93% of 74 patients. Fifty-one percent of patients opted for protocol A, and 49% for protocol B. The complementation rate following incomplete OC was higher in group B (97%) compared with group A (89%), but the results did not reach statistical significance. There were no differences between the rate of complete CCE, the cleansing level, the diagnostic yield or the number of adverse events.

The complementation rate identified in the present study corresponded well with results from recent trials using a first-generation colon capsule. A Spanish study found a complementation rate of incomplete colonoscopies of 85.3% and a diagnostic yield of 45%[23]. A French trial including patients as well with contraindicated as incomplete OC, complete CCE was achieved in 83% of patients. An Italian trial reported a complete PillcamColon2 CCE after incomplete OC in 98% of patients using a separate preparation with Senna tablets, 4 L of PEG and two boosters with NaP and gastrografin[24]. This rate was higher than in our low-volume protocol, but it was accompanied more often by preparation-related complaints (28.0% *vs* 1.4%). Nevertheless, our primary aim to complement colon visualization was achieved in 93% of patients, which was similar to a Greek study that achieved results

Figure 4 Examples of findings at colon capsule endoscopy. A: Biopsy tattoo after optical colonoscopy; B: One of three 4 mm polyps in the ascending colon, confirmed at consecutive balloon enteroscopy as tubular adenoma; C: Significant (7 mm) polyp; D: Cecal adenocarcinoma (confirmed by surgery); E: Fistulating and stenosing Crohn´s disease of the ileum (confirmed by enteroclysis, CT scan, and surgery); F: Irregular Z-line suggestive of Barrett´s esophagus (blue mode image); G: Reflux esophagitis; H: Gastric polyp (consecutive gastroscopy found foveolar hyperplasia and autoimmune gastritis with vitamin B12 deficiency).

using PillCamColon1 in 91% of patients[14]. In the present study, unfavourable anatomy was the reason for terminating the colonoscopy, which was usually performed under propofol sedation, in 92% of patients. In contrast, pain was reported by 45% of patients as

the reason for incomplete colonoscopy in the Italian trial. This selection bias toward unfavourable anatomy might have also influenced the rate of complete consecutive CCEs (65%) in the present study. It may also be relevant when comparing our results to series

Table 3 Details of the 21/74 patients in whom significant polyps/tumors were detected during colon capsule endoscopy

No.	Significance based on	Size of largest polyp (mm)	Number of polyps	Localization of polyps	Farest point reached in OC	All segments with polyps in CCE reached by colonoscopy
1	Size/number	6	5	Rectum	HF	Yes
2	Size	8	2	Sigma	SF	Yes
3	Size/number	8	3	Sigma	AC	Yes
4	Size/number	8	3	2 × Cecum, 1 × AC	TC	No
5	Size	14	1	AC	HF	No
6	Size	26	1	AC	SF	No
7	Size	6	2	AC	Sigma	No
8	Size/number	10	3	2 × AC, 1 × TC	SF	No
9	Size/number	10	3	2 × LC, 1 × AC	Sigma	No
10	Size	10	1	AC	SF	No
11	Size/number	9	3	2 × AC, 1 × LC	HF	No
12	Size	6	1	AC	Sigma	No
13	Size	8	2	1 × AC/1 × LC	HF	No
14	Size	7	1	HF	Sigma	No
15	Number	5	4	AC, TC, 2 × sigma	Sigma	No
16	Size/number	12	4	1 × Cecum, 1 × AC, 1 × LC, 1 × rectum	Sigma	No
17	Number	4	3	1 × Cecum, 2 × rectum	TC	No
18	Number	5	5	2 × AC, 1 × TC, 2 × LC	HF	No
20	Size/number	10	7	4 × AC, 3 × LC	TC	No
20	Size	9	2	2 × Cecum	LC	No
21	Size/number	12	3	AC	LC	No

AC: Ascending colon; HF: Hepatic flexure; TC: Transverse colon; SF: Splenic flexure; LC: Left colon; OC: Optical colonoscopy.

with unselected patients without previously incomplete colonoscopies. In these trials, complete CCE was achieved in 76% of patients using a similar low-volume preparation[18], in 88% of patients using PEG[25], in 76% of patients using NaP[14] and in 98% of patients using a PEG-, NaP- or gastrografin-based regimen[24].

In the present study, colon cleansing using a low-volume preparation was adequate in 67% of patients, and the results were similar in both protocols. These results are comparable to other studies that used PillCamColon1 for incomplete colonoscopies with PEG preparation and NaP boosts (65%)[23], and 60% *vs* 63% in the right and left colon, respectively[14]. Adequate cleansing following use of the PillcamColon1 was observed in 76% of patients following consumption of 1-2 L Moviprep and two NaP boosts in a mixed cohort, including 28% of patients with contraindicated colonoscopy without anatomical problems[19]. A recent Spanish multicentre trial found adequate cleansing levels in 75% of patients using a standard 4 L PEG preparation with a NaP booster for PillCamColon2 after an incomplete colonoscopy[26]. Even in a large trial in a screening population without negative selection towards unfavorable anatomy, CCE was technically insufficient in 9% of patients due to inadequate cleansing or rapid transit of the capsule[27].

Twenty-four percent of our patients (ITT 22%) had additional relevant finding following the CCE, which led to a recommendation for further diagnostics or treatment. Similarly, PillCamColon1 was useful in guiding management after failed OC in a Spanish trial[23].

Most of the significant polyps (86%), including a carcinoma, were found in the AC. Our data confirmed previous observations[5] that CCE can identify relevant lesions in segments not reached by incomplete OC (Table 3).

Device-assisted colonoscopy with either spiral endoscopy and double or single balloons have identical rates, similar to CCE, of complementing incomplete OC of about 90%[28-31]. These flexible endoscopic techniques additionally provide the possibility to directly treat detected lesions, but they require equipment and expertise, and they are restricted to specialized centres. A cap-assisted colonoscopy was also successful in 93% of patients, and one perforation occurred with this technique after failed OC[32].

Computed tomographic colonography (CTC) after incomplete OC has a good diagnostic yield and may be advantageous if extra-colonic findings are considered[33], for example, in the case of tumour. However, CTC detected fewer small polyps than CCE[24]. Furthermore, radiation-free CCE could additionally be used to visualize the Z-line in 60.8% of patients, as previously reported for PillCamColon1 (60%)[34]. In the present trial, eight patients had relevant extra-colonic findings, seven of which were presumably not detectable with CTC.

Capsule retention occurred in one patient, corresponding to a retention rate of 1.4%, which was similar to the rate reported for SBCE[35]. This capsule retention resulted in a new diagnosis of Crohn´s disease and was considered to be diagnostic rather than a complication. However, possible retention must also be considered when applying CCE to complement incomplete OC and should be included as part of the informed consent discussion. Although adhesions were supposed to be the cause of incomplete OC in some patients, no related clinical problems or capsule retention following CCE

were observed.

In conclusion, second-generation CCE using low-volume bowel preparations is useful, well tolerated and is able to detect additional relevant lesions. Risk of retention is as low as in SBCE, but must be considered. Similar results were found in the present study between the two protocols for the complementation rate and presence of significant polyps.

Limitations

Patients could choose between preparation protocols for CCE without randomization. The area reached by the colonoscopy was described, but tattooing was only optional. Long-term follow-up was not part of the present study.

ARTICLE HIGHLIGHTS

Research background

Optical colonoscopy (OC) is the gold standard for visualization of the colon. However, it may be incomplete e.g. due to unfavorable anatomy. Colon capsule endoscopy (CCE) is cleared by the US Food and Drug Administration for patients with previously incomplete OC. Second generation CCE has been shown to have a higher sensitivity for detection of colon polyps than first generation. Low volume bowel prep with Moviprep has been shown to be feasible for CCE.

Research motivation

Bowel preparation for CCE is more extensive than for OC. Thus, we aimed to evaluate, if second generation CCE is feasible using either repeated low volume bowel prep or staying on clear liquids following an incomplete OC.

Research objectives

Main research objective was the ability of CCE to visualize those colon segments not reached by incomplete OC. Secondary objectives were additional diagnostic yield of CCE, rate of complete colon visualization by CCE, cleansing levels, and safety.

Research methods

In this prospective multicenter study 81 patients underwent second generation colon capsule endoscopy with PillCamColon2 after incomplete OC. CCE was performed either the following day (protocol A) after staying on clear liquids and 0.75 L Moviprep in the morning or within 30 d after new split-dose Moviprep (protocol B). Boosts consisted of 0.75 L and 0.25 L Moviprep, and phospho-soda as rescue if the capsule was not excreted after 7 hours.

Research results

Seventy-four patients were finally analyzed per protocol. Of those, cleansing was adequate in 67% of cases and CCE could visualize the colonic segments missed by incomplete colonoscopy in 90% (protocol A) and 97% (protocol B, P = 0.35, n.s.) of the patients. Detection rates were similar with both protocols: Significant polyps and one adenocarcinoma were detected in 24% of cases. Polyps were found predominantly in the right colon (86%) in segments not reached by OC. Extra-colonic findings as reflux esophagitis, suspected Barrett esophagus, upper GI-bleeding, gastric polyps, gastric erosions, and angiectasia were detected in 8 patients. One capsule (1.4%) was retained in the ileum without symptoms and removed during uneventful resection for unknown Crohn`s disease diagnosed as cause of unclear anemia. CCE was well tolerated. One patient suffered from self-limiting vomiting after phospho-soda.

Research conclusions

Second generation CCE using low volume prep is useful to complement incomplete OC, detects additional relevant findings including extra-colonic

lesions, and is well tolerated. CCE is feasible the following day after staying on clear liquids or after new prep within 30 d. Potential risk of capsule retention must be considered.

Research perspectives

Future studies should address improvement of colon cleansing levels and completeness of CCE after incomplete OC. Cost-effectiveness of CCE after incomplete OC should be addressed by future research in comparison with other methods as CT colonoscopy, MR colonoscopy, and device assisted colonoscopy.

REFERENCES

1   **Pox CP**, Altenhofen L, Brenner H, Theilmeier A, Von Stillfried D, Schmiegel W. Efficacy of a nationwide screening colonoscopy program for colorectal cancer. *Gastroenterology* 2012; **142**: 1460-1467.e2 [PMID: 22446606 DOI: 10.1053/j.gastro.2012.03.022]

2   **Brenner H**, Altenhofen L, Katalinic A, Lansdorp-Vogelaar I, Hoffmeister M. Sojourn time of preclinical colorectal cancer by sex and age: estimates from the German national screening colonoscopy database. *Am J Epidemiol* 2011; **174**: 1140-1146 [PMID: 21984657 DOI: 10.1093/aje/kwr188]

3   **Sarwar S**, Anwar MM, Ryan B, O'Morain C. Colonoscopy completion rates - are we prepared for a national screening programme? *Ir Med J* 2007; **100**: 585-587 [PMID: 18196881]

4   **Koido S**, Ohkusa T, Nakae K, Yokoyama T, Shibuya T, Sakamoto N, Uchiyama K, Arakawa H, Osada T, Nagahara A, Watanabe S, Tajiri H. Factors associated with incomplete colonoscopy at a Japanese academic hospital. *World J Gastroenterol* 2014; **20**: 6961-6967 [PMID: 24944489 DOI: 10.3748/wjg.v20.i22.6961]

5   **Neerincx M**, Terhaar sive Droste JS, Mulder CJ, Räkers M, Bartelsman JF, Loffeld RJ, Tuynman HA, Brohet RM, van der Hulst RW. Colonic work-up after incomplete colonoscopy: significant new findings during follow-up. *Endoscopy* 2010; **42**: 730-735 [PMID: 20669092 DOI: 10.1055/s-0030-1255523]

6   **Dafnis G**, Granath F, Påhlman L, Ekbom A, Blomqvist P. Patient factors influencing the completion rate in colonoscopy. *Dig Liver Dis* 2005; **37**: 113-118 [PMID: 15733524 DOI: 10.1016/j.dld.2004.09.015]

7   **Shah HA**, Paszat LF, Saskin R, Stukel TA, Rabeneck L. Factors associated with incomplete colonoscopy: a population-based study. *Gastroenterology* 2007; **132**: 2297-2303 [PMID: 17570204 DOI: 10.1053/j.gastro.2007.03.032]

8   **Loffeld RJ**, van der Putten AB. The completion rate of colonoscopy in normal daily practice: factors associated with failure. *Digestion* 2009; **80**: 267-270 [PMID: 19923819 DOI: 10.1159/000236030]

9   **Hansel SL**, Prechel JA, Horn B, Crowell MD, DiBaise JK. Observational study of the frequency of use and perceived usefulness of ancillary manoeuvres to facilitate colonoscopy completion. *Dig Liver Dis* 2009; **41**: 812-816 [PMID: 19467939 DOI: 10.1016/j.dld.2009.03.010]

10   **Tsai MS**, Su YH, Liang JT, Lai HS, Lee PH. Patient factors predicting the completion of sedation-free colonoscopy. *Hepatogastroenterology* 2008; **55**: 1606-1608 [PMID: 19102351]

11   **Crispin A**, Birkner B, Munte A, Nusko G, Mansmann U. Process quality and incidence of acute complications in a series of more than 230,000 outpatient colonoscopies. *Endoscopy* 2009; **41**: 1018-1025 [PMID: 19856246 DOI: 10.1055/s-0029-1215214]

12   **Britton EJ**, Sidhu S, Geraghty J, Psarelli E, Sarkar S. The 5-year outcome of patients having incomplete colonoscopy. *Colorectal Dis* 2015; **17**: 298-303 [PMID: 25605376 DOI: 10.1111/codi.12901]

13   **Spada C**, Hassan C, Galmiche JP, Neuhaus H, Dumonceau JM, Adler S, Epstein O, Gay G, Pennazio M, Rex DK, Benamouzig R, de Franchis R, Delvaux M, Devière J, Eliakim R, Fraser C, Hagenmuller F, Herrerias JM, Keuchel M, Macrae F, Munoz-Navas M, Ponchon T, Quintero E, Riccioni ME, Rondonotti E, Marmo R, Sung JJ, Tajiri H, Toth E, Triantafyllou K, Van Gossum A, Costamagna G; European Society of Gastrointestinal Endoscopy.

Colon capsule endoscopy: European Society of Gastrointestinal Endoscopy (ESGE) Guideline. *Endoscopy* 2012; **44**: 527-536 [PMID: 22389230 DOI: 10.1055/s-0031-1291717]

14 **Triantafyllou K**, Viazis N, Tsibouris P, Zacharakis G, Kalantzis C, Karamanolis DG, Ladas SD. Colon capsule endoscopy is feasible to perform after incomplete colonoscopy and guides further workup in clinical practice. *Gastrointest Endosc* 2014; **79**: 307-316 [PMID: 24060522 DOI: 10.1016/j.gie.2013.07.061]

15 **Van Gossum A**, Munoz-Navas M, Fernandez-Urien I, Carretero C, Gay G, Delvaux M, Lapalus MG, Ponchon T, Neuhaus H, Philipper M, Costagna G, Riccioni ME, Spada C, Petruzziello L, Fraser C, Postgate A, Fitzpatrick A, Hagenmüller F, Keuchel M, Schoofs N, Devière J. Capsule endoscopy versus colonoscopy for the detection of polyps and cancer. *N Engl J Med* 2009; **361**: 264-270 [PMID: 19605831 DOI: 10.1056/NEJMoa0806347]

16 **Adler S**, Hassan C, Metzger Y, Sompolinsky Y, Spada C. Accuracy of automatic detection of small-bowel mucosa by second-generation colon capsule endoscopy. *Gastrointest Endosc* 2012; **76**: 1170-1174 [PMID: 23025975 DOI: 10.1016/j.gie.2012.07.034]

17 **Spada C**, Pasha SF, Gross SA, Leighton JA, Schnoll-Sussman F, Correale L, González Suárez B, Costamagna G, Hassan C. Accuracy of First- and Second-Generation Colon Capsules in Endoscopic Detection of Colorectal Polyps: A Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol* 2016; **14**: 1533-1543.e8 [PMID: 27165469 DOI: 10.1016/j.cgh.2016.04.038]

18 **Hartmann D**, Keuchel M, Philipper M, Gralnek IM, Jakobs R, Hagenmüller F, Neuhaus H, Riemann JF. A pilot study evaluating a new low-volume colon cleansing procedure for capsule colonoscopy. *Endoscopy* 2012; **44**: 482-486 [PMID: 22275051 DOI: 10.1055/s-0031-1291611]

19 **Pioche M**, de Leusse A, Filoche B, Dalbiès PA, Adenis Lamarre P, Jacob P, Gaudin JL, Coulom P, Letard JC, Borotto E, Duriez A, Chabaud JM, Crampon D, Gincul R, Levy P, ben-Soussan E, Garret M, Lapuelle J, Saurin JC. Prospective multicenter evaluation of colon capsule examination indicated by colonoscopy failure or anesthesia contraindication. *Endoscopy* 2012; **44**: 911-916 [PMID: 22893133 DOI: 10.1055/s-0032-1310008]

20 **Eliakim R**, Yassin K, Niv Y, Metzger Y, Lachter J, Gal E, Sapoznikov B, Konikoff F, Leichtmann G, Fireman Z, Kopelman Y, Adler SN. Prospective multicenter performance evaluation of the second-generation colon capsule compared with colonoscopy. *Endoscopy* 2009; **41**: 1026-1031 [PMID: 19967618 DOI: 10.1055/s-0029-1215360]

21 **Spada C**, Hassan C, Marmo R, Petruzziello L, Riccioni ME, Zullo A, Cesaro P, Pilz J, Costamagna G. Meta-analysis shows colon capsule endoscopy is effective in detecting colorectal polyps. *Clin Gastroenterol Hepatol* 2010; **8**: 516-522 [PMID: 20215066 DOI: 10.1016/j.cgh.2010.02.018]

22 **Triantafyllou K**, Tsibouris P, Kalantzis C, Papaxoinis K, Kalli T, Kalantzis N, Ladas SD. PillCam Colon capsule endoscopy does not always complement incomplete colonoscopy. *Gastrointest Endosc* 2009; **69**: 572-576 [PMID: 19231502 DOI: 10.1016/j.gie.2008.10.047]

23 **Alarcón-Fernández O**, Ramos L, Adrián-de-Ganzo Z, Gimeno-García AZ, Nicolás-Pérez D, Jiménez A, Quintero E. Effects of colon capsule endoscopy on medical decision making in patients with incomplete colonoscopies. *Clin Gastroenterol Hepatol* 2013; **11**: 534-540.e1 [PMID: 23078891 DOI: 10.1016/j.cgh.2012.10.016]

24 **Spada C**, Hassan C, Barbaro B, Iafrate F, Cesaro P, Petruzziello L, Minelli Grazioli L, Senore C, Brizi G, Costamagna I, Alvaro G, Iannitti M, Salsano M, Ciolina M, Laghi A, Bonomo L, Costamagna G. Colon capsule versus CT colonography in patients with incomplete colonoscopy: a prospective, comparative trial. *Gut* 2015; **64**: 272-281 [PMID: 24964317 DOI: 10.1136/gutjnl-2013-306550]

25 **Spada C**, Hassan C, Munoz-Navas M, Neuhaus H, Deviere J, Fockens P, Coron E, Gay G, Toth E, Riccioni ME, Carretero C, Charton JP, Van Gossum A, Wientjes CA, Sacher-Huvelin S, Delvaux M, Nemeth A, Petruzziello L, de Frias CP, Mayershofer R, Amininejad L, Dekker E, Galmiche JP, Frederic M, Johansson GW, Cesaro P, Costamagna G. Second-generation colon capsule endoscopy compared with colonoscopy. *Gastrointest Endosc* 2011; **74**: 581-589.e1 [PMID: 21601200 DOI: 10.1016/j.gie.2011.03.1125]

26 **Nogales Ó**, García-Lledó J, Luján M, Nicolás D, Juanmartiñena JF, González-Suárez B, Sánchez Ceballos F, Couto I, Olmedo J, Garfia C, Carretero C, Fernández Urién I, Rodríguez S, Asteinza M, Olivencia F, Masedo Á, Muñoz-Navas M, Merino B, González Asanza C. Therapeutic impact of colon capsule endoscopy with PillCam™ COLON 2 after incomplete standard colonoscopy: a Spanish multicenter study. *Rev Esp Enferm Dig* 2017; **109**: 322-327 [PMID: 28229607 DOI: 10.17235/reed.2017.4369/2016]

27 **Rex DK**, Adler SN, Aisenberg J, Burch WC Jr, Carretero C, Chowers Y, Fein SA, Fern SE, Fernandez-Urien Sainz I, Fich A, Gal E, Horlander JC Sr, Isaacs KL, Kariv R, Lahat A, Leung WK, Malik PR, Morgan D, Papageorgiou N, Romeo DP, Shah SS, Waterman M. Accuracy of capsule colonoscopy in detecting colorectal polyps in a screening population. *Gastroenterology* 2015; **148**: 948-957.e2 [PMID: 25620668 DOI: 10.1053/j.gastro.2015.01.025]

28 **Becx MC**, Al-Toma A. Double-balloon endoscopy: an effective rescue procedure after incomplete conventional colonoscopy. *Eur J Gastroenterol Hepatol* 2014; **26**: 519-522 [PMID: 24584269 DOI: 10.1097/MEG.0000000000000067]

29 **Dzeletovic I**, Harrison ME, Pasha SF, Crowell MD, Decker GA, Gurudu SR, Leighton JA. Comparison of single- versus double-balloon assisted-colonoscopy for colon examination after previous incomplete standard colonoscopy. *Dig Dis Sci* 2012; **57**: 2680-2686 [PMID: 22615017 DOI: 10.1007/s10620-012-2227-z]

30 **Schembre DB**, Ross AS, Gluck MN, Brandabur JJ, McCormick SE, Lin OS. Spiral overtube-assisted colonoscopy after incomplete colonoscopy in the redundant colon. *Gastrointest Endosc* 2011; **73**: 515-519 [PMID: 21353848 DOI: 10.1016/j.gie.2010.11.047]

31 **Sulz MC**, Frei R, Semadeni GM, Sawatzki M, Borovicka J, Meyenberger C. The role of single-balloon colonoscopy for patients with previous incomplete standard colonoscopy: Is it worth doing it? *Surg Endosc* 2016; **30**: 1876-1882 [PMID: 26183958 DOI: 10.1007/s00464-015-4405-5]

32 **Lee YT**, Hui AJ, Wong VW, Hung LC, Sung JJ. Improved colonoscopy success rate with a distally attached mucosectomy cap. *Endoscopy* 2006; **38**: 739-742 [PMID: 16673307 DOI: 10.1055/s-2006-925238]

33 **Pullens HJ**, van Leeuwen MS, Laheij RJ, Vleggaar FP, Siersema PD. CT-colonography after incomplete colonoscopy: what is the diagnostic yield? *Dis Colon Rectum* 2013; **56**: 593-599 [PMID: 23575398 DOI: 10.1097/DCR.0b013e3182781668]

34 **Schoofs N**, Devière J, Van Gossum A. PillCam colon capsule endoscopy compared with colonoscopy for colorectal tumor diagnosis: a prospective pilot study. *Endoscopy* 2006; **38**: 971-977 [PMID: 17058159 DOI: 10.1055/s-2006-944835]

35 **Liao Z**, Gao R, Xu C, Li ZS. Indications and detection, completion, and retention rates of small-bowel capsule endoscopy: a systematic review. *Gastrointest Endosc* 2010; **71**: 280-286 [PMID: 20152309 DOI: 10.1016/j.gie.2009.09.031]

Baishideng®

World Journal of

Gastroenterology

Contents

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Fabio Grizzi, PhD, Assistant Professor, Department of Immunology and Inflammation, Humanitas Clinical and Research Hospital, Rozzano 20089, Italy

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 642 experts in gastroenterology and hepatology from 59 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

EDITORS FOR THIS ISSUE

Responsible Assistant Editor: *Xiang Li*
Responsible Electronic Editor: *Shu-Yu Yin*
Proofing Editor-in-Chief: *Lian-Sheng Ma*

Responsible Science Editor: *Ruo-Yu Ma*
Proofing Editorial Office Director: *Ze-Mao Gong*

Positioning of old and new biologicals and small molecules in the treatment of inflammatory bowel diseases

Jason Reinglas, Lorant Gonczi, Zsuzsanna Kurt, Talat Bessissow, Peter L Lakatos

Jason Reinglas, Talat Bessissow, Peter L Lakatos, Department of Gastroenterology, McGill University Health Center, Montreal, Québec H4A 3J1, Canada

Lorant Gonczi, Zsuzsanna Kurti, Peter L Lakatos, First Department of Medicine, Semmelweis University, H-1083, Budapest, Koranyi S. 2A, Hungary

ORCID number: Jason Reinglas (0000-0001-5455-260X); Lorant Gonczi (0000-0002-8819-6460); Zsuzsanna Kurti (0000-0001-8671-6576); Talat Bessissow (0000-0003-2610-1910); Peter L Lakatos (0000-0002-3948-6488).

Correspondence to: Peter L Lakatos, DSc, MD, PhD, Professor, Division of Gastroenterology, Montreal General Hospital C7-200, McGill University Health Center, 1650 Cedar Avenue, Montreal, Quebec H3G 1A4, Canada. kislakpet99@gmail.com
Telephone: +36-1-2100278

Fax: +36-1-3130250

Abstract

The past decade has brought substantial advances in the management of inflammatory bowel diseases (IBD). The introduction of tumor necrosis factor (TNF) antagonists, evidence for the value of combination therapy, the recognition of targeting lymphocyte trafficking and activation as a viable treatment, and the need for early treatment of high-risk patients are all fundamental concepts for current modern IBD treatment algorithms. In this article, authors review the existing data on approved biologicals and small molecules as well as provide insight on the current positioning of approved therapies. Patient stratification for the selection of specific therapies, therapeutic targets and patient monitoring will be discussed as well. The therapeutic armamentarium for IBD is expanding as novel and more targeted therapies become available. In the absence of comparative trials, positioning these agents is becoming difficult. Emerging concepts for the future will include an emphasis on the development of algorithms which will facilitate a greater understanding of the positioning of novel biological drugs and small molecules in order to best tailor therapy to the patient. In the interim, anti-TNF therapy remains an important component of IBD therapy with the most real-life evidence and should be considered as first-line therapy in patients with complicated Crohn's disease and in acute-severe ulcerative colitis. The safety and efficacy of these 'older' anti-TNF therapies can be optimized by adhering to therapeutic algorithms which combine clinical and objective markers of disease severity

and response to therapy.

Key words: Inflammatory bowel disease; Small molecule; Positioning; Biologic; Therapeutic

Core tip: Anti-tumor necrosis factor therapy should be considered as first-line therapy in patients with complicated Crohn's disease and in acute-severe ulcerative colitis. Beyond these specific circumstances, the positioning of novel biologics and small molecules depends on the patient's medical history, preference and disease phenotype. The efficacy and safety of using immunomodulatory therapy can be enhanced by adhering to therapeutic algorithms and using a 'treat-to-target' approach. The risks for adverse events due to poor disease control outweigh the risks associated with early aggressive therapy. In the setting of clinical and biochemical remission, following at least 6 mo of combined immunosuppressive therapy, consideration can be made to withdrawing thiopurine therapy in the correct patient with close follow-up.

INTRODUCTION

Therapeutic trials for inflammatory bowel disease (IBD) began nearly 100 years after the first case report of IBD was published by Sir Samuel Wilks in 1859 who used the term "ulcerative colitis (UC)" to describe a condition similar to what is understood as UC today[1]. Approximately 10 years following the original study by Sir Sidney Truelove which revealed the efficacy of corticosteroid therapy in UC, the first clinical trial evaluating steroids in Crohn's disease (CD) was conducted in 1966 by Jones and Lennard-Jones[2]. Prior to these landmark trials, the treatment of IBD was limited to supportive care and surgical intervention.

Knowledge regarding the adverse effects of chronic steroid therapy in UC ultimately led to the first positive double blind randomized controlled trial (RCT) evaluating the efficacy of sulfasalazine in 1962[3,4]. Unfortunately, many patients were unable to tolerate the side-effects from sulfasalazine which prompted additional studies to uncover the active ingredient, 5ASA[5]. Since, 5ASA has repeatedly demonstrated its efficacy and improved safety profile as compared to sulfasalazine in mild to moderate UC[6-8]. In contrast, 5ASA therapy has been abandoned in CD due to its inability to prevent quiescent

disease relapse[9]. As steroid-refractory disease became more prevalent, reports on the use of ciclosporin began appearing and the first successful trials were conducted in 1989 and 1994 for steroid resistant severe CD and UC, respectively[10,11]. Due to ciclosporin's narrow therapeutic window, alternative steroid-sparing agents such as thiopurines were investigated. Although they have demonstrated fair efficacy in IBD, it may take up to 3-6 mo for them to reach their full therapeutic effect thereby limiting their potential as a strong induction agent[12]. Despite their slow onset of action and risks, thiopurines may be used strategically to reduce immunogenicity associated with biologic therapy and augment the rate of remission[13,14] Budesonide, a corticosteroid which undergoes significant first-pass metabolism in the liver resulting in low systemic exposure, has also established its position in the therapeutic armamentarium since Rutgeerts *et al*[15]'s original study demonstrating its non-inferiority to prednisolone therapy for CD patients in 1994. Budesonide has since repeatedly demonstrated its efficacy and safety making it the preferred means of inducing remission in patients with mild Crohn's ileitis[16]. A newer formulation with a delayed release (budesonide-MMX©) can be efficacious in moderate UC as well[17].

Alongside the advent of new biological therapies, the therapeutic approach has evolved over the past decade to include the use of objective markers of disease severity and response to therapy in tandem with the historical clinical scores[18,19]. In this article, authors review the existing data and provide a rationale for the positioning of the 'old' and 'new' biologicals and small molecules. Strategies for the use of available therapies based on recent guidelines will be reviewed.

CD

Anti-tumor necrosis factor
Infliximab: Four years after the FDA approved the use of infliximab in CD, the first large RCT; ACCENT I, was published in 2002 which evaluated infliximab maintenance therapy in 573 patients with a CDAI of at least 220 whom had responded well to an initial infusion of infliximab[20]. At the 30 and 54 wk follow-up, patients receiving infliximab maintenance therapy were more likely to be in remission (CDAI < 150) as compared to those without maintenance therapy (30 wk: OR = 2.7, 95%CI: 1.6-4.6) with a similar incidence of infection across all groups[20]. Besides demonstrating infliximab's efficacy, this study also provided a rationale for dose escalation in patients losing response to therapy[21]. Although effective for luminal disease, it was unclear if infliximab would also be effective for fistulising disease, thus the ACCENT II trial was published 2 years later which included 306 patients with one or more draining abdominal or perianal fistulas of at least 3 mo duration[22]. In this trial, the patients who were undergoing infliximab maintenance therapy demonstrated a significant fistula response wherein 36% (*vs* 23%, *P* = 0.009) had

complete resolution of fistula draining at 54 wk[22]. Additionally, ACCENT Ⅱ demonstrated a significant reduction in the requirement for hospitalization and surgery due to fistulising disease (8.6% *vs* 18.9%, *P* < 0.05)[23]. Early initiation of infliximab was further supported in a large study conducted by the GETAID group which evaluated the use of dual therapy *vs* monotherapy over 52 wk in 113 steroid-dependant CD patients[13]. Both GETAID and ACCENT-Ⅰ studies identified incongruence amongst endoscopy and clinical scores, such as the CDAI. In a sub-study of ACCENT-Ⅰ, 18% of moderate to severe CD patients as determined by the CDAI score had no active CD on endoscopy[24]. This prompted a rationale to include more objective end points and markers of disease severity (*e.g.*, CRP and mucosal healing) in future studies, as was included in the Study of Biologic and Immunomodulator Naive Patients in Crohn's Disease (SONIC) conducted in 2010[14]. In this landmark RCT involving 508 biologic- and immunosuppressive-naïve patients, the superiority of infliximab over azathioprine as well as the therapeutic advantage of combining therapies over monotherapy with either infliximab or azathioprine alone at the 30 and 50 wk follow-ups was demonstrated.[14].

Adalimumab: In an attempt to possibly reduce the immunogenic responses induced by chimeric antibodies, such as infliximab which contains 25% mouse sequences, adalimumab was designed as the first fully human monoclonal antibody against tumor necrosis factor (TNF)-alpha[25]. The results of three pivotal trials (CLASSIC-Ⅰ, CHARM and GAIN) established regulatory approval of adalimumab for the induction and maintenance of remission of CD in 2007. CLASSIC-Ⅰ was the first human trial to evaluate induction of remission using adalimumab in 299 moderate to severe CD patients naïve to anti-TNF therapy[26]. A linear dose-response curve was appreciated at the 4 wk follow-up, with the greatest clinical remission rate associated with the highest dose studied (160 mg and 80 mg at weeks 0 and 2, respectively)[25]. As a ceiling effect was not achieved, it is unclear if higher dosing would be more efficacious, studies evaluating this are underway. The use of adalimumab as a second line induction agent following the failure of infliximab due to intolerance or poor response was evaluated in the GAIN trial which included 325 patients who had either lost response or become intolerant to infliximab[27]. At the 4 wk follow-up, 21% (34 of 159) of patients in the adalimumab group *vs* 7% (12 of 166) of those in the placebo group achieved clinical remission.

The efficacy of adalimumab for maintenance therapy was evaluated in the CLASSIC-Ⅱ and CHARM studies. CLASSIC-Ⅱ followed up with 276 patients from the CLASSIC-Ⅰ study at 56 wk after randomizing patients to receive maintenance dosing or placebo. A greater proportion of patients receiving adalimumab 40 mg SC weekly or biweekly were in remission as compared to those receiving placebo (83% and 79% *vs* 44%,

respectively)[28]. Additionally, although most patients responded to therapy within the first week, some patients only responded to therapy after week 12[28]. This suggests that an observational period may need to occur prior to modifying therapy in patients who do not respond to induction following 1 wk. In the largest open-label study, CHARM enrolled 854 patients in order to evaluate the efficacy of adalimumab for induction and maintenance in CD patients not responding to alternative immunosuppressive therapy, including those whom had failed infliximab[29]. Although the induction dose of adalimumab was half of that provided in the CLASSIC trials, the response rate was similar. At week 56, biweekly and weekly dosing was equally effective at maintaining remission as compared to placebo (36% and 41% *vs* 12%, respectively). Of note, a greater proportion of patients receiving placebo discontinued treatment due to adverse events as compared to those receiving adalimumab[29]. This suggests the risks of complications associated with poorly controlled disease outweigh the risks associated with therapy. To corroborate the findings from the previous studies demonstrating clinical remission, the EXTEND trial conducted in 2012 which involved 135 patients with moderate to severe ileocolonic CD demonstrated a trend towards mucosal healing with adalimumab at week 12 as compared to placebo (27% *vs* 13%, respectively) as well as a significant difference at week 52 (24% *vs* 0%, respectively (*P* < 0.001))[30]. Again, this suggests 12 wk may not be sufficient in all patients to determine response to therapy.

Certolizumab: Certolizumab pegol is a pegylated humanized monoclonal antibody Fab' fragment linked to polyethylene glycol that has a high affinity to tumor necrosis factor alpha[31]. Certolizumab was proposed as a potential alternative to infliximab due to its ease of delivery (SC as oppose to infusion) and longer half-life which may reduce the need for frequent dosing and risk for immunogenicity, theoretically[32,33]. The risks for side effects were presumed to be lower due to the lack of an Fc region which would be responsible for activating the compliment pathway leading to cellular apoptosis[32,33]. The largest phase Ⅱ trial in 2005 by Schreiber *et al*[33] in 292 patients with moderate to severe CD demonstrated a significant dose-response relationship with clinical benefit demonstrated up until week 10, then lost significance at week 12 which was presumed to be secondary to greater placebo rates in patients with lower CRP values[33]. The potential placebo effect was addressed in the PRECISE-Ⅰ trial which stratified 662 patients with moderate to severe CD based on their CRP prior to randomization to treatment groups[31]. Although response rates at week 6 and 26 were found to be modestly significant, induction of remission rates were not. However, in patients responding to certolizumab, maintenance of remission was successfully demonstrated in the PRECISE-Ⅱ and PRECISE-Ⅲ follow-up trials through 5 years[34,35]. The MUSIC trial conducted in 2013 confirmed certolizumab's efficacy with respect to mucosal

healing following 54 wk of therapy after evaluating 89 patients with active endoscopic disease (ulceration in ⩾ 2 intestinal segments with a Crohn's Disease Endoscopic Index of Severity (CDEIS) score ⩾ 8 points)[36]. As early as week 10, endoscopic remission was achieved in 37% of patients.

Anti-integrin
Natalizumab: Natalizumab blocks the adhesion and subsequent migration of leukocytes from circulation into the gut by binding alpha-4 integrin which is expressed on all circulating leukocytes except neutrophils. Originally designed for multiple sclerosis patients, natalizumab demonstrated good efficacy for induction and maintenance of remission for CD in a large meta-analysis which included 5 trials[37]. The largest trials to be performed were ENACT-I, ENACT-II and ENCORE. ENACT-I included 905 patients with CD randomized to either placebo or natalizumab induction groups[38]. Although there was a subtle but significant difference in the response rate favoring natalizumab (56 percent and 49 percent, respectively), there was no difference in remission rates between groups for induction. ENACT-II included 339 responders to natalizumab from ENACT-I and randomized them to maintenance therapy every 4 wk or placebo[38]. In contrast to the first trial, significantly higher rates of remission occurred through 36 wk as compared to placebo (44% *vs* 26%). Induction of remission was reassessed in the ENCORE study which included 509 patients with CD evaluated through 3 induction doses over 8 wk. At week 12, a greater proportion of patients on natalizumab were in remission as compared to placebo, 28% *vs* 16% respectively[39].

Although natalizumab demonstrated good efficacy in luminal CD, concerns related to serious infection surfaced. In an open-label extension of the ENACT-II trial, one patient died from JC virus-associated progressive multifocal leukoencephalopathy (PML)[40]. The association with PML and natalizumab was described in two other case reports on patients receiving treatment for multiple sclerosis[41,42]. Since the estimated risk for PML is 1 per 1000 patients, JC virus antibody testing should be considered if natalizumab will be used in IBD.

Vedolizumab: Vedolizumab reduces lymphocyte migration into the gut by antagonizing the $\alpha_4\beta_7$ integrin mediated reactions. In contrast to natalizumab it does not act on $\alpha_4\beta_1$ integrin, which is involved in brain lymphocyte trafficking, thus may have lower risk for PML[43]. Efficacy for its use as an induction and maintenance agent in CD was demonstrated in the GEMINI-II trial[44]. In the induction component of the trial, 368 patients were randomized to placebo or vedolizumab and 747 patients received open-label vedolizumab. Approximately 50% of all patients had failed at least one anti-TNF prior to enrolling in the study. Although clinical remission was achieved in a significantly greater proportion of patients taking vedolizumab as compared to placebo at week 6

(14.5% *vs* 6.8%, respectively), there was no significant difference in CDAI scores greater than 100 (CDAI-100 score) or CRP levels between groups. However, nearly twice as many patients in the vedolizumab maintenance groups were in clinical remission as compared to the placebo group (39% *vs* 21.6% respectively). Significant differences in favor of maintenance therapy over placebo were demonstrated in the CRP and the CDAI-100 score. Fistulization also improved as compared to placebo in the small group of patients on vedolizumab every 8 wk (*n* = 17) but not in the small group taking vedolizumab every 4 wk[44]. Acknowledging that subjects recruited for this study had likely more aggressive disease than the aforementioned biologic-naïve anti-TNF studies discussed, vedolizumab is efficacious for luminal and possibly fistulising disease but may not provide as effective and efficient induction as compared to anti-TNF therapy. This has also been supported in network meta-analyses[45]. As such, if rapid induction is required then physicians prescribing vedolizumab should be aware of the potentially slower onset of action and consideration for the concomitant use of faster-acting induction agents (*e.g.*, corticosteroids) to bridge the patient symptomatically.

A common reason for using vedolizumab as first line treatment in IBD is the assumption of the reduced risk for infection given the attenuation of the immune response is localized to the gut. This has been previously supported in a review which included six trials evaluating the use of vedolizumab in UC and CD (2380 patients with 4811 person-years of vedolizumab exposure)[46]. Within this study however, 16 patients with CD in the vedolizumab group developed clostridium difficile infection as compared to none in the placebo group. Additionally, more patients on vedolizumab had gastroenteritis and developed tuberculosis infection (despite negative tuberculosis screening at enrollment). In the aforementioned GEMINI-II trial, vedolizumab also had a higher rate of infections (44.1% *vs* 40.2%), and serious infections (5.5% *vs* 3.0%) as compared to placebo[44]. Head to head trials are needed to better describe the risk for infection in patients taking vedolizumab as compared to other biologics.

Ustekinumab: IL-12 p35-p40 and IL-23 p19-p40 are two proinflammatory heterodimeric cytokines that are induced in the inflamed mucosa of CD patients[47,48]. Ustekinumab is a human monoclonal IgG_{1k} antibody which blocks the P40 sub-unit of IL-12 and IL-23 on T cells, natural killer and antigen presenting cells[49]. Originally successful in the treatment for plaque psoriasis and psoriatic arthritis, ustekinumab demonstrated its efficacy for CD in the UNITI trials which included 1300 CD patients with moderate to severe disease[50]. UNITI-I included 741 patients whom had failed anti-TNF therapy due to non-response or intolerance. The induction component of the trial revealed a significantly better clinical response in the ustekinumab treatment groups as com-

pared to placebo (34% *vs* 22%, respectively). UNITI-II included 628 patients whom were anti-TNF naïve but failed conventional immunosuppressive therapy due to poor response or intolerance. The UNITI-II cohort also had a significant improvement in their CDAI scores for induction by approximately 25% as compared to placebo. Patients receiving maintenance therapy every 8 wk and every 12 wk demonstrated a significantly greater remission rate at week 44 as compared to placebo (53% and 49% *vs* 36%, respectively). Of note, the secondary analyses demonstrated a non-significant difference in CDAI scores compared to placebo in the UNITI-I group as compared to the UNITI-II group, albeit the trend still favored ustekinumab therapy[50]. Lack of significance is most likely due to a lack of power to properly evaluate the difference amongst sub-groups, however this trend is expected; patients in UNITI-I have more refractory disease thus less likely to respond to ustekinumab as compared to the biologic-naïve patients in UNITI-II. Significant improvements in fecal calprotectin and CRP were also noted and able to be seen as early as 3 wk supporting its usefulness in acute severe flares.

UC

Anti-TNF agents

Infliximab: The first two large-scale studies to assess the therapeutic potential of infliximab were the ACT 1 and ACT 2 trials published in 2005, prior to this, biologic therapy for UC was not established[51]. ACT 1 evaluated 364 patients with moderate to severe UC following their induction and maintenance dosing until 54 wk. ACT 2 evaluated the same number of patients and maintained the same induction, maintenance and follow-up regimen as ACT 1 except maintenance dosing ceased after 22 wk. Nearly 60% of patients in both cohorts were steroid dependent. In both studies, a significant clinical response was demonstrated with remission occurring in approximately 35% and 31% of patients taking infliximab as compared to 15% and 6% of patients on placebo at week 8 in ACT 1 and ACT 2 studies, respectively. Sustained remission was achieved over the study period in approximately 20% of patients on infliximab as compared to 5% of patients in the placebo group. Additionally, a greater proportion of patients were able to be weaned off their steroids following the initiation of infliximab. Mucosal healing, considered to be the greatest risk factor for malignancy, was markedly improved throughout the study period and significantly better than placebo as early as week 8, approximately 60% *vs* 30% respectively. No difference between the two doses prescribed, 10 mg/kg and 5 mg/kg, was identified with respect to efficacy[51].

Given the toxicity associated with cyclosporine and limited therapies available, GETAID compared the efficacy of infliximab against cyclosporine in an open-label RCT involving 115 patients with severe ulcerative colitis whom had failed high dose intravenous steroid therapy. The results were positive for both agents with no

significant difference in treatment failure or side effects between the infliximab and the ciclosporin groups (54% *vs* 60%, respectively)[52].

Adalimumab: Five years following the approval for infliximab use in UC, adalimumab became the second biologic approved for use in UC based on the results from the ULTRA trials. ULTRA 1 utilized two different induction regimens (160/80 mg *vs* 80/40 mg SC at weeks 0 and 2 followed by 40 mg every 2 wk) to evaluate if adalimumab was effective in 186 moderate to severe UC patients[53]. At week 8, 19% *vs* 9% were in remission in the 160/80 mg group as compared to placebo, respectively. As noted in the CD trials, a ceiling effect was not achieved thus the optimal dose is still under investigation. ULTRA 2, which included 518 patients with moderate to severe UC, was conducted to evaluate the long-term efficacy of adalimumab as a maintenance agent[53]. Following 1 year, remission was achieved in 17% of patients on regular maintenance dosing as compared to 9% of patients in the placebo group. Similarly, mucosal healing was also higher in the adalimumab group as compared to placebo at both week 8 and 52 follow-up intervals, 41% and 25% *vs* 32% and 15%, respectively. This study also demonstrated that biologic naïve patients were more likely to achieve clinical remission as compared to patients previously on infliximab (Week 8: 21% *vs* 9% and Week 52: 22% *vs* 10%, respectively), which highlights prior biologic use as a potential risk factor for difficult to treat or aggressive disease. Long-term maintenance therapy using adalimumab was further evaluated over 4 years in ULTRA 1 and 2 trials as well as in an open-label study (ULTRA 3)[54]. With respect to patients observed as nonresponder imputation (NRI), 25% and 28% of the 199 patients from ULTRA 1 and 2 whom were still on adalimumab at the 4 year follow-up maintained clinical remission and mucosal healing respectively. In contrast, the ULTRA 3 open-label trial demonstrated clinical remission and mucosal healing rates to be considerably greater (64% and 60%, respectively), albeit difficult to compare in the absence of randomization.

Golimumab: Golimumab is a fully human monoclonal immunoglobulin delivered subcutaneously which targets a unique epitope on the TNF molecule as compared to infliximab and adalimumab. The PURSUIT trials which evaluated 1064 biologic naive patients with moderate to severe UC were responsible for establishing regulatory approval for it in 2014. The induction trial, PURSUIT-SC, revealed a significantly greater proportion of patients in clinical remission following 6 wk using 200/100 mg and 400/200 mg induction doses as compared to placebo, 51% and 55% *vs* 30% respectively[55]. The extension of this trial, PURSUIT-M, which included 464 patients with moderate to severe UC whom had responded favorably to golimumab in the induction trial also demonstrated greater efficacy than placebo at maintaining clinical remission following 54 wk. At study end, 42% of patients

taking golimumab 100 mg every 4 wk were found to be in clinical remission as compared to 27% of patients taking placebo[56]. The rate of mucosal healing was significantly greater for patients taking golimumab in both the induction and maintenance studies, the differences were able to be appreciated as early as 2 wk. Golimumab, although not formally assessed in clinical trials, has been reported to be efficacious as a second and third-line anti-TNF agent in real life settings[57].

Anti-integrin agent

Vedolizumab: GEMINI-1 evaluated the efficacy of vedolizumab in a treatment resistant group of 895 moderate to severe UC patients (approximately 40% of patients failed ⩾ 1 anti-TNF therapy)[58]. In the induction phase of the trial, 17% of patients taking vedolizumab were in clinical remission as compared to 5% of patients taking placebo by week 6. Mucosal healing was also nearly twice as apparent in patients taking vedolizumab as compared to placebo (41% *vs* 25% respectively). At 52 wk, clinical remission was maintained in approximately 44% of patients taking vedolizumab as compared to 16% of patients on placebo. No significant difference was identified between treatment groups receiving every 4 or 8 wk dosing regimens. In contrast to GEMINI-Ⅱ for CD, there was no difference in infection rates in the treatment group as compared to placebo[44,58].

SMALL MOLECULES

JAK inhibitors

Tofacitinib: Tofacitinib is a new oral medication which suppresses cytokine signalling in mucosal immune cells by inhibiting janus kinase's 1 and 3 (JAK 1 and 3). The oral route of administration and ability to target multiple cytokine pathways makes JAK inhibitors an attractive therapeutic option.

Although the efficacy for tofacitinib has not been established in CD yet, it has been established in UC as demonstrated by the OCTAVE trials[59]. Of the 905 patients with moderate to severe UC randomized to treatment in the induction trials, approximately 18% achieved clinical remission as compared to 6% of patients in the placebo group at 8 wk. Onset to effect was rapid, with improvements in their partial mayo score demonstrated as early as 2 wk. Although over 50% of patients within the induction groups had prior exposure to anti-TNF therapy, the treatment effect was similar in comparison to patients whom were biologic naïve despite OCTAVE's more stringent criteria for clinical remission as compared to the aforementioned trials (*i.e.*, partial mayo rectal bleeding subscore of 0). The OCTAVE-Sustain extension trial, which included 593 patients who had a clinical response to induction therapy, also demonstrated good maintenance of remission after 52 wk in both 5 mg and 10 mg twice daily treatment groups as compared to placebo (34% and 41% *vs* 11%, respectively). Mucosal healing and steroid-free remission was achieved and

maintained in a similar proportion of patients. With respect to adverse events, serious infections occurred more frequently in the induction but not maintenance trial. However, herpes zoster infection did occur more frequently in the tofacitinib 10mg maintenance group as compared to placebo[59]. Of note, tofacitinib received a recommendation for the treatment of UC by the GIDAC-FDA in March 2018 a final decision is anticipated by June 2018[60].

BIOSIMILARS

According to the FDA, a biosimilar is defined as a biological product that is highly similar to the reference product notwithstanding minor differences in clinically inactive components which result in no clinically meaningful differences in the purity, safety and efficacy of the product[61]. The use of biologic anti-inflammatory medications is increasing and the cost has become a significant economic burden on many national healthcare systems around the world[62]. In Canada, the growth of Canadian sales of biologic anti-inflammatory drugs has nearly doubled since 2010. The top-selling biologic, remicade (infliximab), has cost the Canadian Government $224 million in 2015 and $4.8 billion since it was approved 10 years ago. Based on a Market Intelligence Report published by Health Canada, the use of a biosimilar such as Inflectra could have resulted in a $41.7 million reduction in drug expenditures in 2015[62]. Several biosimilars to remicade (flixabi, inflectra, remsima) and adalimumab (cyltezo and imraldi) have already been approved for use in IBD.

Infliximab-dyyb (or CT-P13), was the first biosimilar for remicade (infliximab) to be approved and has the greatest amount of 'real world' observational data evaluating its efficacy and safety[63]. Infliximab-dyyb was first approved in South Korea and thereafter in Europe in 2013 following the results of two large randomized and double-blind clinical studies evaluating its safety and efficacy in rheumatoid arthritis as compared to remicade, PLANETRA and PLANETAS[64,65]. No significant differences were found with respect to safety, efficacy and immunogenicity thus it was approved for use in all labelled indications remicade was approved for. However, small retrospective studies in IBD have demonstrated mixed results[66-68]. A larger prospective nationwide multi-center study performed in Hungary involving 126 CD and 84 UC patients reported excellent induction rates[69]. At week 14, 81% of patients with Crohn's disease and 78% of patients with ulcerative colitis had a clinical response (CDAI reduction > 70) and 54% and 59% respectively, were in clinical remission (CDAI < 150). Comparable results were also seen in another large observational cohort study including 313 CD and 234 UC patients[70]. Response rates at 8 wk were greater than 90% for all patient groups, including patients whom switched from remicade to infliximab-dyyb. At week 24, response rates were 73.7%, 62.2% and 78.9% for biologic naïve,

Medication	Route of administration (IV, SC, PO)	Approved dose
		Table 1 Currently approved biologic treatments for inflammatory bowel diseases[16,117,118]
Infliximab	IV	Induction: 5-10 mg/kg (weeks 0, 2, and 6)
		Maintenance: 5-10 mg/kg every 4-8 wk
Adalimumab	SC	Induction: 160 mg (week 0), 80 mg (week 2)
		Maintenance: 40 mg every 7-14 d
Golimumab	SC	Induction: 200 mg (week 0), 100 mg (week 2)
		Maintenance: 100 mg every 4 wk
Certolizumab	SC	Induction: 400 mg (weeks 0, 2, and 4)
		Maintenance: 400 mg every 4 wk
Vedolizumab	IV	Induction: 300 mg (weeks 0, 2, and 6)
		Maintenance: 300 mg every 4-8 wk
Ustekinumab	IV	Induction:
	SC	< 55 kg: 260 mg
		55-85 kg: 390 mg
		> 85 kg: 520 mg
		Maintenance: 90 mg every 8 wk

pre-exposed and switched respectively. The efficacy, immunogenicity and safety profiles in both studies were considered comparable to that of the originator drug infliximab.

To date, studies which have evaluated switching from originator to biosimilar have been largely positive[71,72]. The longest evaluation period occurred over 52 wk in the NOR-SWITCH study which was a randomised, non-inferiority, double-blind, phase 4 trial involving 482 patients across 40 Norwegian centres with various inflammatory diseases maintained in remission on infliximab for at least 6 mo. Of the 482 patients, 155 (32%) and 93 (19%) were CD and UC respectively[73]. At study end, there was no difference in disease worsening, safety or immunogenicity amongst any of the groups. Although switching therapies in the setting of controlled disease would be a reasonable option and is supported by the evidence as well as the European Crohns and Colitis Organization; switching in the setting of failing the originator drug would be ill-advised[71]. Ben-Horin *et al*[74] studied the cross reactivity of antibodies to remicade and infliximab-dyyb in 125 patients with IBD and healthy individuals as negative controls. They demonstrated that anti-remicade antibodies recognize and inhibit infliximab-dyyb as well. These results suggested that there was similar immunogenicity and shared immunodominant epitopes. Although this supported the safety of biosimilars and the use of the same assay as the originator drug to detect antibodies, this study also supported not using the biosimilar in the setting of originator failure[71,74,75].

Evolution of treatment strategies of IBD and positioning currently approved biologics and small molecules in clinical practice

As the therapeutic armamentarium for IBD continues to expand, so follows the complexity associated with managing IBD patients in clinical practice. The needs for algorithms are required in order to assist health care practitioners determine the relative positioning of each agent and their use in combination with other therapies. Until the results of head to head biologic and small molecule trials become available, we can only speculate the positioning of therapeutic agents based on the current available literature as summarized in this section (Table 1).

Positioning the 'old' biologics: Anti-TNFs first, alone or in combination?

As newer and more targeted therapies in IBD become available, questions related to maintaining anti-TNF agents as first line therapy arise. Based on decades of data, anti-TNFs currently provide the best long-term evidence of efficacy in CD and UC, with a known safety profile. They are effective for both induction and maintenance therapy, decrease corticosteroid exposure and promote sustained mucosal healing[76,77]. The most important safety concern is the risk of serious infection. However, in younger patients without co-existing medical problems, this risk is fairly low[78].

Comparing efficacy of TNF inhibitors is difficult due to the lack of high-quality, head-to-head trials (Table 2). Network meta-analyses indirectly comparing anti-TNF agents have reported mixed results[45,79-81]. Based on 'real world' data, an analysis of retrospective and comparative effectiveness database studies revealed subtle differences regarding hospitalisation and surgery rates as well as the steroid sparing effect between infliximab and adalimumab, favouring infliximab at currently recommended doses. Of note, clinical trials of higher-dose adalimumab for both UC and CD are currently underway[82,83].

Deciding between which anti-TNF agent to use depends on the clinical circumstances, treatment history and patient preference. In the absence of head-to-head comparisons, there exists few specific scenarios in which the evidence supports the use of specific anti-TNF agents. In the setting of a hospitalized patient with severe UC, only infliximab has demonstrated its efficacy as a 'rescue' therapy[84]. Patients with perianal disease can benefit from either infliximab or adalimumab, albeit the evidence is based on a post-hoc analysis for adalimumab and lacking for other anti-TNF agents[23,85]. Golimumab

Table 2 Biologic agents which have demonstrated efficacy in inflammatory bowel diseases and rheumatology

	Mechanism of action	UC	CD	[2]Fistulization	Ankylosing Spondylitis	Psoriasis
Anti-TNF						
[1]Infliximab[20,22,51,119]	Chimeric monoclonal antibody	x	x	x	x	x
Adalimumab[26,28,54,120,121]	Fully human monoclonal antibody	x	x	x	x	x
Certolizumab[31,122,123]	Pegylated humanized monoclonal antibody Fab' fragment		x	+/-	x	x
Golimumab[57,122,124]	Fully human monoclonal antibody	x			x	x
Anti-integrin						
[4]Natalizumab[39]	Chimeric monoclonal antibody against α4 integrin		x			
[3]Vedolizumab[46,96]	Chimeric monoclonal antibody against α4β7 integrin	x	x	+/-		
Ustekinumab[50,125,126]	Fully human monoclonal antibody against P40 sub-unit of IL-12 and IL-23		x	+/-	x	x

[1]Infliximab is the only biologic which has been evaluated to be an effective 'rescue' agent. Evidence is lacking for the remaining biologics; [2]Improvement in fistulizing disease was evaluated as a primary outcome only in infliximab. Efficacy was otherwise determined indirectly from secondary outcomes, subgroup analyses and small scale studies for the remaining biologics; [3]Consider the use of vedolizumab as a first-line biologic agent in patients at high risk for infectious complications. Vedolizumab has a slower onset of action (approximately 6-8 wk) as compared to alternate biologics; [4]Use of natalizumab is contraindicated if the patient is JC virus antibody positive due to the risk of progressive multifocal leukoencephalopathy. UD: Ulcerative colitis; CD: Crohn's disease.

has demonstrated efficacy in UC as a second or third line anti-TNF agent in small cohorts of patients but not for CD. Similarly, certolizumab can be considered in the same context for CD but lacks evidence for UC. Ease of administration may influence one's decision thus patients who would rather less frequent dosing may prefer the IV infusion infliximab as compared to the other anti-TNF agents which are delivered SC by the patient.

The relatively high costs of anti-TNFs and the expiration of patents have triggered the development of biosimilar monoclonal antibodies. Multiple regulatory agencies have approved the use of biosimilars in IBD based on extrapolation of data on safety and efficacy. Since then, real-word data and randomised controlled trials on switching from originator to biosimilar infliximab has shown similar results in terms of efficacy and safety[72]. Following the introduction of vedoluzimab and ustekinumab, anti-TNF therapy may not be the first-line biologic agent in all IBD patients. However, the lower cost of biosimilars probably makes the use of anti-TNF agents still very attractive.

Optimizing the efficacy of the initial anti-TNF therapy prior to switching to another biologic, either in or out of class, is a critical principle when managing IBD patients. Studies have repeatedly demonstrated that patients failing their first biologic have poorer outcomes following initiation of their second or third biologic[50,58]. The ability to differentiate the cause for a loss of response to anti-TNF therapy has been facilitated with therapeutic drug monitoring (TDM)[86]. Based on TDM results, an educated decision regarding dose optimization and switching in or out of class can now be determined[16,87]. However, the frequency of TDM is still up for debate. Few retrospective studies have demonstrated benefit with proactive TDM[88,89]. The recent multicentre prospective RCT involving 167 patients with active CD, TAILORIX, demonstrated that there was no benefit in patients receiving infliximab dose escalation based on TDM as compared to clinical scoring[89]. Although more patients in the

clinical dose escalation group received dose escalation as compared to the TDM group, thus the benefit seen from the clinical group may be over-inflated. Similarly, the TAXIT study, which was a 1 year RCT involving 178 CD and 85 UC patients performed at a single tertiary referral center, did not find benefit in proactive vs reactive (i.e., symptom based) TDM[88]. However, the results from the TAXIT study should be interpreted with caution since dose optimisation occurred in both groups at study start. Prospective, multi-center studies are needed to further investigate the positioning of TDM.

The decision to initiate combination therapy involves balancing the benefits of improved efficacy and lower immunogenicity of therapy against the heightened risks for infection and malignancy. The SONIC trial revealed the steroid-free remission rate in CD patients at week 26 was significantly greater in the combination azathioprine and infliximab group as compared to infliximab or azathioprine alone (57% vs 44% vs 30%, respectively)[14]. The SUCCESS trial, which was a 16 week RCT involving 239 patients with moderate to severe UC, revealed similar results. Steroid-free remission was achieved in 40% of patients on dual therapy as compared to 22% and 24% on infliximab and azathioprine monotherapy, respectively[90]. Supporting this strategy was the open label prospective DIAMOND study which evaluated 176 Japanese patients with CD over 52 wk. This study demonstrated that the efficacy of using dual therapy was not limited to only infliximab but also to adalimumab. Mucosal healing was significantly better in the combination group as compared to the azathioprine monotherapy group at week 26 (84% vs 64%, respectively)[91]. Although the difference in clinical remission was not significant, likely due to a small cohort and lower thiopurine dosing, a trend was maintained in favor of combination therapy. The infection and serious complication risks were not greater on dual therapy as compared to monotherapy in either of the aforementioned studies. In contrast, the SONIC trial

demonstrated the lowest risk for infection to be present in the dual therapy group (3.9%) as compared to the infliximab or azathioprine monotherapy groups (4.9% and 5.6%, respectively). This suggests that poorly controlled disease is a stronger risk factor for infection instead of intensified immunosuppression. Ultimately, the risk for hepatosplenic T-cell lymphoma (especially in young/adolescent males after 2 years of therapy), myelosuppression and opportunistic infections must be weighted individually[92,93]. Consideration can be made to initiating therapy with both combined thiopurine and anti-TNF therapy than stopping thiopurine therapy after 6 mo in the setting of clinical and biochemical remission and a therapeutic drug level, which has been supported in the literature[94,95].

Positioning 'new' agents: First or second-line?

Vedolizumab has emerged as a first-line agent for induction of remission for moderately active UC patients failing conventional therapy[58]. In CD, clinicians should be aware of the potentially slower onset of action of vedolizumab. Concomitant use of corticosteroids may be necessary during the induction period. For these reasons, anti-TNFs or ustekinumab may be more favourable first line choices in CD patients with severe disease activity at present. There is also no considerable data from RCTs on the efficacy of vedolizumab in fistulizing CD and acute severe UC. Ongoing phase IV trial will determine its effectiveness[96]. Vedolizumab is currently being positioned in some jurisdictions as a second-line biologic agent following anti-TNFs, although the ongoing LOVE studies are evaluating the use of vedolizumab in early vs. late UC and CD[97,98]. Given their effectiveness in the medium to long term and the favourable safety profile, it is expected that gut-selective anti-integrin agents will increasingly be used as maintenance therapy or even as part of a combination biological therapy. A clinical trial evaluating the efficacy of adalimumab, methotrexate and vedolizumab triple combination therapy is ongoing[99].

Ustekinumab is the most recently approved biologic agent for CD[50]. Presently, there is no data available describing its efficacy in UC or fistulising CD. An indirect comparison amongst the anti-TNF and UNITI trials suggests ustekinumab may be safer and have a lower rate of immunogenicity which may make it the preferred biologic for some CD patients[77]. More comprehensive data on efficacy in certain patient subgroups and mucosal healing is needed.

Finally, tofacitinib is a small molecule awaiting final approval for the treatment of UC[59,60]. Their oral route of administration makes them particularly attractive. Their safety profile has been suggested to be similar to that of thiopurines. Due to their mechanism of action, they are not limited by immunogenicity and subsequent loss of response. Their positioning and use as mono- or combination therapy has yet to be elucidated.

The evolution of treatment strategies and objective monitoring: Early aggressive or tailored therapy?

The introduction of highly effective therapies early in the disease course alongside objective patient monitoring can modify the disease trajectory and reduce morbidity. However, it is also important to recognize that approximately 20% of patients with IBD may have an indolent disease course, and available population-based data suggests that approximately half of patients with CD can be symptomatically controlled 10 years after diagnosis[100,101]. Risk stratification can guide early introduction of highly effective therapy in patients with a poor prognosis and prevent overtreatment in low-risk patients. Unfortunately, current patient stratification relies on clinical factors. Most of these are indicators rather than predictors of a complicated disease course (*e.g.*, presence of perianal disease, age < 40 years old at diagnosis and need for steroids during the first flare)[102]. Molecular makers for predicting an aggressive phenotype have yet to be identified but studies are ongoing[103,104].

In the absence of objective predictors for disease severity, studies have attempted to better elucidate the risks and benefits of aggressive therapy. The TOP-DOWN trial was the first to assess and compare different treatment algorithms in IBD[105]. Treatment-naïve early CD patients were randomly assigned to receive early aggressive therapy ('top-down') with an immunosuppressant and anti-TNF agent or less aggressive ('step-up') therapy with steroids and a possible transition to immunosuppressant and biologics if necessary. The authors found that the 'top-down' strategy was more effective than the conventional 'step-up' strategy for achieving corticosteroid-free remission at week 52 (61.5% *vs* 42.2%, *P* = 0.027). Similar conclusions were demonstrated in both the SONIC and UC-SUCCESS trials whereby the efficacy of therapy was improved despite comparable adverse events between groups.

The strengths of objective patient monitoring are becoming more evident as study designs continue to improve and include more objective markers of disease severity and response to therapy. An example is the cluster randomisation trial, REACT[106]. In this trial, 1982 patients with CD were randomized to receive either algorithm-based treatment optimization vs. conventional management (therapeutic decisions based on community physician assessment). The composite endpoint of hospitalization, surgery and serious disease related complications was lower in patients treated with the algorithm-based strategy at 24 mo (27.7% and 35.1%, hazard ratio: 0.73, 95%CI: 0.62 to 0.86, *P* < 0.001), despite no differences in serious drug-related adverse events as compared to the conventional treatment group. In UC, evidence is less straightforward on whether 'top-down' therapy alters the long-term disease outcomes. Although several studies have shown that the severity and extent of UC at diagnosis may have a major impact on the subsequent course of the disease with elevated risks of recurrent hospitalization, colectomy, cancer and mortality[101,107,108]. In a population-based inception cohort from Norway, the extent of disease, need for systemic steroids and high CRP at diagnosis were independently associated with colectomy[109]. Consequently, patients presenting with extensive colitis and signs of severe disease

Table 3 Recommendations for treating to target in Crohn's disease by the International Organization for the Study of Inflammatory Bowel Diseases[19]

Crohn's disease	Ulcerative colitis
The consensus target is a combination of:	
Clinical/[1]PRO remission defined as resolution of abdominal pain and diarrhea or altered bowel habits which should be assessed every 3 mo until resolution then 6-12 mo thereafter.	Clinical/[1]PRO remission defined as resolution of rectal bleeding and diarrhea or altered bowel habits which should be assessed every 3 mo until resolution then 6-12 mo thereafter.
and	and
Endoscopic remission[2] defined as resolution of ulceration at ileocolonoscopy which should be assessed at 6-9 mo intervals during the active phase	Endoscopic remission[2] defined as resolution of friability and ulceration at flexible sigmoidoscopy or colonoscopy[3] which should be assessed at 3 mo intervals during the active phase
Adjunctive measures of disease activity that may be useful in the management of selected patients but are not a treatment target include:	
•Faecal calprotectin	•CRP
	•Faecal calprotectin
	•Histology
Measures of disease activity that are not a target:	
•Histology	•Cross-sectional imaging
•Cross-sectional imaging	

[1]Patient reported outcomes; [2]When endoscopy cannot adequately evaluate inflammation, resolution of inflammation as assessed by cross-sectional imaging can be substituted; [3]While Mayo subscore of 0 may be defined as the target, there is currently insufficient evidence to recommend it in all patients; only Mayo subscore of 0-1 can be systematically recommended in practice.

at diagnosis could benefit from top-down therapy.

'Treat to target', a strategy that uses objective clinical and biochemical outcome measures to assist clinicians in making decisions related to modifying therapy, has been gaining popularity since the REACT study demonstrated that disease activity correlates relatively poorly with objective measures of inflammation, and clinical remission in the absence of mucosal healing may not necessarily decrease the risk of future complications in CD[106]. The CALM study supported this logic as well and demonstrated that early and stringent control of disease using objective markers of inflammation (*e.g.*, CRP and fecal calprotectin) was efficacious and safe in their sample population of 244 patients with CD[18]. Their primary end-point, mucosal healing at 48 wk, was achieved in 46% *vs* 30% of the patients in the 'tight control' group as compared to the 'clinical management' group. Deep, biological and steroid-free remissions were greater in the 'tight control' group as well, whilst the adverse events not significantly different between groups. The recent systematic review and expert opinion of 28 IBD specialists on 'Selecting Therapeutic Targets in Inflammatory Bowel Disease' (STRIDE) also suggested the importance of using objective markers and recommends that therapeutic targets for CD and UC should move away from composite disease activity indices to separate patient-reported outcomes and objective measurements of inflammation (Table 3)[19]. However, the open-label multicentre RCT 'CALM' suggested that biomarkers such as fecal calprotectin be considered as additional targets to therapy in their cohort of 244 patients with active CD. Besides acting as a treatment target, biomarkers can facilitate the monitoring of a patient. For example, elevated c-reactive protein or fecal calprotectin should prompt further endoscopic and/or radiologic evaluation irrespective of clinical scores. Although intensified regimens are efficacious, they are also more likely to encounter difficulties with patient compliance. Additional guidance regarding the use of endoscopic findings as treatment targets will come following the completion of the REACT-II prospective trial.

FUTURE PERSPECTIVES

Data obtained from head to head biologic and small molecule trials will eventually be applied to clinical practice in order to better individualize and optimize therapy. The determination of which therapies can be combined best will be further elucidated as well. For instance, combining anti-TNF therapy with vedolizumab is being evaluated in studies for patients with refractory disease because it combines a rapidly acting systemic agent with a slower acting gut-specific therapy. The development of oral medications with specific targets (*e.g.*, filgotinib) will open the door to a large range of potential therapeutic combinations which will enable therapy to be individualized further[110]. Specific therapies such as antifibrotics, SMAD7 inhibitors, sphingosine 1-phosphate receptor modulators and phosphodiesterase inhibitors are quickly making their way through trial phases and can be expected to hold a place in the IBD armamentarium in the near future[111-113]. As in Oncology, omics will enable us to determine which patients are at greatest risk for a complicated disease course thus provide a rationale for initiating intensified immunotherapy at diagnosis and individualize therapy best[114]. Molecular imaging and pretreatment genetic and biomarker analysis may be able to predict response to a proposed therapy in the future and are currently being investigated[103,104,115,116].

CONCLUSION

As the quality of trial designs improved over the decades, so followed our understanding of IBD. This has enabled

us to tailor therapy and develop effective treatment algorithms using clinical symptomps/PROS, biomarkers and endoscopic indices to help guide therapy. Anti-TNF therapy remains an important component of IBD therapy with the most real-life evidence and should be considered as first-line therapy in patients with complicated CD and in acute-severe UC. Novel mono- and combination therapies have only begun to be approved and offer the ability to tailor therapy further. However, clinicians will be faced with important challenges in defining the optimal use of these new therapies and their relative position in treatment algorithms. The next generation of clinical trials will need to ascertain the answers to these questions.

REFERENCES

1   **Wilks S**. Morbid appearances in the intestines of Miss Bankes. *Lond Med Gaz* 1859; **2**: 264-265

2   **Jones JH**, Lennard-Jones JE. Corticosteroids and corticotrophin in the treatment of Crohn's disease. *Gut* 1966; **7**: 181-187 [PMID: 4286707 DOI: 10.1136/gut.7.2.181]

3   **De Dombal FT**. Ulcerative colitis: definition, historical background, aetiology, diagnosis, naturel history and local complications. *Postgrad Med J* 1968; **44**: 684-692 [PMID: 5705372 DOI: 10.1136/pgmj.44.515.684]

4   **Baron JH**, Connell AM, Lennard-Jones JE, Jones FA. Sulphasalazine and salicylazosulphadimidine in ulcerative colitis. *Lancet* 1962; **1**: 1094-1096 [PMID: 13865153 DOI: 10.1016/S0140-6736(62)92080-9]

5   **Azad Khan AK**, Piris J, Truelove SC. An experiment to determine the active therapeutic moiety of sulphasalazine. *Lancet* 1977; **2**: 892-895 [PMID: 72239 DOI: 10.1016/S0140-6736(77)90831-5]

6   **Feagan BG**, Macdonald JK. Oral 5-aminosalicylic acid for induction of remission in ulcerative colitis. *Cochrane Database Syst Rev* 2012; **10**: CD000543 [PMID: 23076889 DOI: 10.1002/14651858.CD000543.pub3]

7   **Wang Y**, Parker CE, Feagan BG, MacDonald JK. Oral 5-aminosalicylic acid for maintenance of remission in ulcerative colitis. *Cochrane Database Syst Rev* 2016; CD000544 [PMID: 27158764 DOI: 10.1002/14651858.CD000544.pub4]

8   **Marshall JK**, Thabane M, Steinhart AH, Newman JR, Anand A, Irvine EJ. Rectal 5-aminosalicylic acid for induction of remission in ulcerative colitis. *Cochrane Database Syst Rev* 2010; CD004115 [PMID: 20091560 DOI: 10.1002/14651858.CD004115.pub2]

9   **Ford AC**, Kane SV, Khan KJ, Achkar JP, Talley NJ, Marshall JK, Moayyedi P. Efficacy of 5-aminosalicylates in Crohn's disease: systematic review and meta-analysis. *Am J Gastroenterol* 2011; **106**: 617-629 [PMID: 21407190 DOI: 10.1038/ajg.2011.71]

10   **Lichtiger S**, Present DH, Kornbluth A, Gelernt I, Bauer J, Galler G, Michelassi F, Hanauer S. Cyclosporine in severe ulcerative colitis refractory to steroid therapy. *N Engl J Med* 1994; **330**: 1841-1845 [PMID: 8196726 DOI: 10.1056/NEJM199406303302601]

11   **Brynskov J**, Freund L, Rasmussen SN, Lauritsen K, de Muckadell OS, Williams N, MacDonald AS, Tanton R, Molina F, Campanini MC. A placebo-controlled, double-blind, randomized trial of cyclosporine therapy in active chronic Crohn's disease. *N Engl J Med* 1989; **321**: 845-850 [PMID: 2671739 DOI: 10.1056/NEJM198909283211301]

12   **Axelrad JE**, Roy A, Lawlor G, Korelitz B, Lichtiger S. Thiopurines and inflammatory bowel disease: Current evidence and a historical perspective. *World J Gastroenterol* 2016; **22**: 10103-10117 [PMID: 28028358 DOI: 10.3748/wjg.v22.i46.10103]

13   **Lémann M**, Mary JY, Duclos B, Veyrac M, Dupas JL, Delchier JC, Laharie D, Moreau J, Cadiot G, Picon L, Bourreille A, Sobahni I, Colombel JF; Groupe d'Etude Thérapeutique des Affections Inflammatoires du Tube Digestif (GETAID). Infliximab plus azathioprine for steroid-dependent Crohn's disease patients: a randomized placebo-controlled trial. *Gastroenterology* 2006; **130**: 1054-1061 [PMID: 16618399 DOI: 10.1053/j.gastro.2006.02.014]

14   **Colombel JF**, Sandborn WJ, Reinisch W, Mantzaris GJ, Kornbluth A, Rachmilewitz D, Lichtiger S, D'Haens G, Diamond RH, Broussard DL, Tang KL, van der Woude CJ, Rutgeerts P; SONIC Study Group. Infliximab, azathioprine, or combination therapy for Crohn's disease. *N Engl J Med* 2010; **362**: 1383-1395 [PMID: 20393175 DOI: 10.1056/NEJMoa0904492]

15   **Rutgeerts P**, Löfberg R, Malchow H, Lamers C, Olaison G, Jewell D, Danielsson A, Goebell H, Thomsen OO, Lorenz-Meyer H. A comparison of budesonide with prednisolone for active Crohn's disease. *N Engl J Med* 1994; **331**: 842-845 [PMID: 8078530 DOI: 10.1056/NEJM199409293311304]

16   **Gomollón F**, Dignass A, Annese V, Tilg H, Van Assche G, Lindsay JO, Peyrin-Biroulet L, Cullen GJ, Daperno M, Kucharzik T, Rieder F, Almer S, Armuzzi A, Harbord M, Langhorst J, Sans M, Chowers Y, Fiorino G, Juillerat P, Mantzaris GJ, Rizzello F, Vavricka S, Gionchetti P; ECCO. 3rd European Evidence-based Consensus on the Diagnosis and Management of Crohn's Disease 2016: Part 1: Diagnosis and Medical Management. *J Crohns Colitis* 2017; **11**: 3-25 [PMID: 27660341 DOI: 10.1093/ecco-jcc/jjw168]

17   **Abdalla MI**, Herfarth H. Budesonide for the treatment of ulcerative colitis. *Expert Opin Pharmacother* 2016; **17**: 1549-1559 [PMID: 27157244 DOI: 10.1080/14656566.2016.1183648]

18   **Colombel JF**, Panaccione R, Bossuyt P, Lukas M, Baert F, Vaňásek T, Danalioglu A, Novacek G, Armuzzi A, Hébuterne X, Travis S, Danese S, Reinisch W, Sandborn WJ, Rutgeerts P, Hommes D, Schreiber S, Neimark E, Huang B, Zhou Q, Mendez P, Petersson J, Wallace K, Robinson AM, Thakkar RB, D'Haens G. Effect of tight control management on Crohn's disease (CALM): a multicentre, randomised, controlled phase 3 trial. *Lancet* 2018; **390**: 2779-2789 [PMID: 29096949 DOI: 10.1016/S0140-6736(17)32641-7]

19   **Peyrin-Biroulet L**, Sandborn W, Sands BE, Reinisch W, Bemelman W, Bryant RV, D'Haens G, Dotan I, Dubinsky M, Feagan B, Fiorino G, Gearry R, Krishnareddy S, Lakatos PL, Loftus EV Jr, Marteau P, Munkholm P, Murdoch TB, Ordás I, Panaccione R, Riddell RH, Ruel J, Rubin DT, Samaan M, Siegel CA, Silverberg MS, Stoker J, Schreiber S, Travis S, Van Assche G, Danese S, Panes J, Bouguen G, O'Donnell S, Pariente B, Winer S, Hanauer S, Colombel JF. Selecting Therapeutic Targets in Inflammatory Bowel Disease (STRIDE): Determining Therapeutic Goals for Treat-to-Target. *Am J Gastroenterol* 2015; **110**: 1324-1338 [PMID: 26303131 DOI: 10.1038/ajg.2015.233]

20   **Hanauer SB**, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, Rutgeerts P; ACCENT I Study Group. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* 2002; **359**: 1541-1549 [PMID: 12047962 DOI: 10.1016/S0140-6736(02)08512-4]

21   **Rutgeerts P**, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, Hanauer SB. Comparison of scheduled and episodic treatment strategies of infliximab in Crohn's disease. *Gastroenterology* 2004; **126**: 402-413 [PMID: 14762776 DOI: 10.1053/j.gastro.2003.11.014]

22   **Sands BE**, Anderson FH, Bernstein CN, Chey WY, Feagan BG, Fedorak RN, Kamm MA, Korzenik JR, Lashner BA, Onken JE, Rachmilewitz D, Rutgeerts P, Wild G, Wolf DC, Marsters PA, Travers SB, Blank MA, van Deventer SJ. Infliximab maintenance therapy for fistulizing Crohn's disease. *N Engl J Med* 2004; **350**: 876-885 [PMID: 14985485 DOI: 10.1056/NEJMoa030815]

23   **Lichtenstein GR**, Yan S, Bala M, Blank M, Sands BE. Infliximab maintenance treatment reduces hospitalizations, surgeries, and procedures in fistulizing Crohn's disease. *Gastroenterology* 2005; **128**: 862-869 [PMID: 15825070 DOI: 10.1053/j.gastro.2005.01.048]

24   **Rutgeerts P**, Diamond RH, Bala M, Olson A, Lichtenstein GR, Bao W, Patel K, Wolf DC, Safdi M, Colombel JF, Lashner B, Hanauer SB. Scheduled maintenance treatment with infliximab is superior to episodic treatment for the healing of mucosal ulceration associated with Crohn's disease. *Gastrointest Endosc* 2006; **63**: 433-42; quiz 464 [PMID: 16500392 DOI: 10.1016/j.gie.2005.08.011]

25   **Cassinotti A**, Ardizzone S, Porro GB. Adalimumab for the treatment of Crohn's disease. *Biologics* 2008; **2**: 763-777 [PMID: 19707457]

26   **Hanauer SB**, Sandborn WJ, Rutgeerts P, Fedorak RN, Lukas M, MacIntosh D, Panaccione R, Wolf D, Pollack P. Human anti-tumor necrosis factor monoclonal antibody (adalimumab) in Crohn's disease: the CLASSIC-I trial. *Gastroenterology* 2006; **130**: 323-333; quiz 591 [PMID: 16472588 DOI: 10.1053/j.gastro.2005.11.030]

27   **Sandborn WJ**, Rutgeerts P, Enns R, Hanauer SB, Colombel JF, Panaccione R, D'Haens G, Li J, Rosenfeld MR, Kent JD, Pollack PF. Adalimumab induction therapy for Crohn disease previously treated with infliximab: a randomized trial. *Ann Intern Med* 2007; **146**: 829-838 [PMID: 17470824 DOI: 10.7326/0003-4819-146-12-200706190-00159]

28   **Sandborn WJ**, Hanauer SB, Rutgeerts P, Fedorak RN, Lukas M, MacIntosh DG, Panaccione R, Wolf D, Kent JD, Bittle B, Li J, Pollack PF. Adalimumab for maintenance treatment of Crohn's disease: results of the CLASSIC II trial. *Gut* 2007; **56**: 1232-1239 [PMID: 17299059 DOI: 10.1136/gut.2006.106781]

29   **Colombel JF**, Sandborn WJ, Rutgeerts P, Enns R, Hanauer SB, Panaccione R, Schreiber S, Byczkowski D, Li J, Kent JD, Pollack PF. Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial. *Gastroenterology* 2007; **132**: 52-65 [PMID: 17241859 DOI: 10.1053/j.gastro.2006.11.041]

30   **Rutgeerts P**, Van Assche G, Sandborn WJ, Wolf DC, Geboes K, Colombel JF, Reinisch W; EXTEND Investigators, Kumar A, Lazar A, Camez A, Lomax KG, Pollack PF, D'Haens G. Adalimumab induces and maintains mucosal healing in patients with Crohn's disease: data from the EXTEND trial. *Gastroenterology* 2012; **142**: 1102-1111.e2 [PMID: 22326435 DOI: 10.1053/j.gastro.2012.01.035]

31   **Sandborn WJ**, Feagan BG, Stoinov S, Honiball PJ, Rutgeerts P, Mason D, Bloomfield R, Schreiber S; PRECISE 1 Study Investigators. Certolizumab pegol for the treatment of Crohn's disease. *N Engl J Med* 2007; **357**: 228-238 [PMID: 17634458 DOI: 10.1056/NEJMoa067594]

32   **Nesbitt A**, Fossati G, Bergin M, Stephens P, Stephens S, Foulkes R, Brown D, Robinson M, Bourne T. Mechanism of action of certolizumab pegol (CDP870): in vitro comparison with other anti-tumor necrosis factor alpha agents. *Inflamm Bowel Dis* 2007; **13**: 1323-1332 [PMID: 17636564 DOI: 10.1002/ibd.20225]

33   **Schreiber S**, Rutgeerts P, Fedorak RN, Khaliq-Kareemi M, Kamm MA, Boivin M, Bernstein CN, Staun M, Thomsen OØ, Innes A; CDP870 Crohn's Disease Study Group. A randomized, placebo-controlled trial of certolizumab pegol (CDP870) for treatment of Crohn's disease. *Gastroenterology* 2005; **129**: 807-818 [PMID: 16143120 DOI: 10.1053/j.gastro.2005.06.064]

34   **Schreiber S**, Khaliq-Kareemi M, Lawrance IC, Thomsen OØ, Hanauer SB, McColm J, Bloomfield R, Sandborn WJ; PRECISE 2 Study Investigators. Maintenance therapy with certolizumab pegol for Crohn's disease. *N Engl J Med* 2007; **357**: 239-250 [PMID: 17634459 DOI: 10.1056/NEJMoa062897]

35   **Lichtenstein GR**, Thomsen OØ, Schreiber S, Lawrance IC, Hanauer SB, Bloomfield R, Sandborn WJ; Precise 3 Study Investigators. Continuous therapy with certolizumab pegol maintains remission of patients with Crohn's disease for up to 18 months. *Clin Gastroenterol Hepatol* 2010; **8**: 600-609 [PMID: 20117244 DOI: 10.1016/j.cgh.2010.01.014]

36   **Hébuterne X**, Lémann M, Bouhnik Y, Dewit O, Dupas JL, Mross M, D'Haens G, Mitchev K, Ernault É, Vermeire S, Brixi-Benmansour H, Moreels TG, Mary JY, Marteau P, Colombel JF. Endoscopic improvement of mucosal lesions in patients with moderate to severe ileocolonic Crohn's disease following treatment with certolizumab pegol. *Gut* 2013; **62**: 201-208 [PMID: 22525883 DOI: 10.1136/gutjnl-2012-302262]

37   **Ford AC**, Sandborn WJ, Khan KJ, Hanauer SB, Talley NJ, Moayyedi P. Efficacy of biological therapies in inflammatory bowel disease: systematic review and meta-analysis. *Am J Gastroenterol* 2011; **106**: 644-659, quiz 660 [PMID: 21407183 DOI: 10.1038/ajg.2011.73]

38   **Sandborn WJ**, Colombel JF, Enns R, Feagan BG, Hanauer SB, Lawrance IC, Panaccione R, Sanders M, Schreiber S, Targan S, van Deventer S, Goldblum R, Despain D, Hogge GS, Rutgeerts P; International Efficacy of Natalizumab as Active Crohn's Therapy (ENACT-1) Trial Group; Evaluation of Natalizumab as Continuous Therapy (ENACT-2) Trial Group. Natalizumab induction and maintenance therapy for Crohn's disease. *N Engl J Med* 2005; **353**: 1912-1925 [PMID: 16267322 DOI: 10.1056/NEJMoa043335]

39   **Targan SR**, Feagan BG, Fedorak RN, Lashner BA, Panaccione R, Present DH, Spehlmann ME, Rutgeerts PJ, Tulassay Z, Volfova M, Wolf DC, Hernandez C, Bornstein J, Sandborn WJ; International Efficacy of Natalizumab in Crohn's Disease Response and Remission (ENCORE) Trial Group. Natalizumab for the treatment of active Crohn's disease: results of the ENCORE Trial. *Gastroenterology* 2007; **132**: 1672-1683 [PMID: 17484865 DOI: 10.1053/j.gastro.2007.03.024]

40   **Van Assche G**, Van Ranst M, Sciot R, Dubois B, Vermeire S, Noman M, Verbeeck J, Geboes K, Robberecht W, Rutgeerts P. Progressive multifocal leukoencephalopathy after natalizumab therapy for Crohn's disease. *N Engl J Med* 2005; **353**: 362-368 [PMID: 15947080 DOI: 10.1056/NEJMoa051586]

41   **Kleinschmidt-DeMasters BK**, Tyler KL. Progressive multifocal leukoencephalopathy complicating treatment with natalizumab and interferon beta-1a for multiple sclerosis. *N Engl J Med* 2005; **353**: 369-374 [PMID: 15947079 DOI: 10.1056/NEJMoa051782]

42   **Langer-Gould A**, Atlas SW, Green AJ, Bollen AW, Pelletier D. Progressive multifocal leukoencephalopathy in a patient treated with natalizumab. *N Engl J Med* 2005; **353**: 375-381 [PMID: 15947078 DOI: 10.1056/NEJMoa051847]

43   **Soler D**, Chapman T, Yang LL, Wyant T, Egan R, Fedyk ER. The binding specificity and selective antagonism of vedolizumab, an anti-alpha4beta7 integrin therapeutic antibody in development for inflammatory bowel diseases. *J Pharmacol Exp Ther* 2009; **330**: 864-875 [PMID: 19509315 DOI: 10.1124/jpet.109.153973]

44   **Sandborn WJ**, Feagan BG, Rutgeerts P, Hanauer S, Colombel JF, Sands BE, Lukas M, Fedorak RN, Lee S, Bressler B, Fox I, Rosario M, Sankoh S, Xu J, Stephens K, Milch C, Parikh A; GEMINI 2 Study Group. Vedolizumab as induction and maintenance therapy for Crohn's disease. *N Engl J Med* 2013; **369**: 711-721 [PMID: 23964933 DOI: 10.1056/NEJMoa1215739]

45   **Singh S**, Garg SK, Pardi DS, Wang Z, Murad MH, Loftus EV Jr. Comparative efficacy of biologic therapy in biologic-naïve patients with Crohn disease: a systematic review and network meta-analysis. *Mayo Clin Proc* 2014; **89**: 1621-1635 [PMID: 25441399 DOI: 10.1016/j.mayocp.2014.08.019]

46   **Colombel JF**, Sands BE, Rutgeerts P, Sandborn W, Danese S, D'Haens G, Panaccione R, Loftus EV Jr, Sankoh S, Fox I, Parikh A, Milch C, Abhyankar B, Feagan BG. The safety of vedolizumab for ulcerative colitis and Crohn's disease. *Gut* 2017; **66**: 839-851 [PMID: 26893500 DOI: 10.1136/gutjnl-2015-311079]

47   **Fuss IJ**, Becker C, Yang Z, Groden C, Hornung RL, Heller F, Neurath MF, Strober W, Mannon PJ. Both IL-12p70 and IL-23 are synthesized during active Crohn's disease and are down-regulated by treatment with anti-IL-12 p40 monoclonal antibody. *Inflamm Bowel Dis* 2006; **12**: 9-15 [PMID: 16374252 DOI: 10.1097/01.MIB.0000194183.92671.b6]

48   **Monteleone G**, Biancone L, Marasco R, Morrone G, Marasco O, Luzza F, Pallone F. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. *Gastroenterology* 1997; **112**: 1169-1178 [PMID: 9098000 DOI: 10.1016/S0016-5085(97)70128-8]

49   **Benson JM**, Peritt D, Scallon BJ, Heavner GA, Shealy DJ, Giles-Komar JM, Mascelli MA. Discovery and mechanism of ustekinumab: a human monoclonal antibody targeting interleukin-12 and interleukin-23 for treatment of immune-mediated disorders. *MAbs* 2011; **3**: 535-545 [PMID: 22123062 DOI: 10.4161/mabs.3.6.17815]

50   **Feagan BG**, Sandborn WJ, Gasink C, Jacobstein D, Lang Y, Friedman JR, Blank MA, Johanns J, Gao LL, Miao Y, Adedokun OJ, Sands BE, Hanauer SB, Vermeire S, Targan S, Ghosh S, de Villiers WJ, Colombel JF, Tulassay Z, Seidler U, Salzberg BA, Desreumaux P, Lee SD, Loftus EV Jr, Dieleman LA, Katz S, Rutgeerts P;

UNITI-IM-UNITI Study Group. Ustekinumab as Induction and Maintenance Therapy for Crohn's Disease. *N Engl J Med* 2016; **375**: 1946-1960 [PMID: 27959607 DOI: 10.1056/NEJMoa1602773]

51 **Rutgeerts P**, Sandborn WJ, Feagan BG, Reinisch W, Olson A, Johanns J, Travers S, Rachmilewitz D, Hanauer SB, Lichtenstein GR, de Villiers WJ, Present D, Sands BE, Colombel JF. Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med* 2005; **353**: 2462-2476 [PMID: 16339095 DOI: 10.1056/NEJMoa050516]

52 **Laharie D**, Bourreille A, Branche J, Allez M, Bouhnik Y, Filippi J, Zerbib F, Savoye G, Nachury M, Moreau J, Delchier JC, Cosnes J, Ricart E, Dewit O, Lopez-Sanroman A, Dupas JL, Carbonnel F, Bommelaer G, Coffin B, Roblin X, Van Assche G, Esteve M, Färkkilä M, Gisbert JP, Marteau P, Nahon S, de Vos M, Franchimont D, Mary JY, Colombel JF, Lémann M; Groupe d'Etudes Thérapeutiques des Affections Inflammatoires Digestives. Ciclosporin versus infliximab in patients with severe ulcerative colitis refractory to intravenous steroids: a parallel, open-label randomised controlled trial. *Lancet* 2012; **380**: 1909-1915 [PMID: 23063316 DOI: 10.1016/S0140-6736(12)61084-8]

53 **Sandborn WJ**, van Assche G, Reinisch W, Colombel JF, D'Haens G, Wolf DC, Kron M, Tighe MB, Lazar A, Thakkar RB. Adalimumab induces and maintains clinical remission in patients with moderate-to-severe ulcerative colitis. *Gastroenterology* 2012; **142**: 257-65.e1-3 [PMID: 22062358 DOI: 10.1053/j.gastro.2011.10.032]

54 **Colombel JF**, Sandborn WJ, Ghosh S, Wolf DC, Panaccione R, Feagan B, Reinisch W, Robinson AM, Lazar A, Kron M, Huang B, Skup M, Thakkar RB. Four-year maintenance treatment with adalimumab in patients with moderately to severely active ulcerative colitis: Data from ULTRA 1, 2, and 3. *Am J Gastroenterol* 2014; **109**: 1771-1780 [PMID: 25155227 DOI: 10.1038/ajg.2014.242]

55 **Sandborn WJ**, Feagan BG, Marano C, Zhang H, Strauss R, Johanns J, Adedokun OJ, Guzzo C, Colombel JF, Reinisch W, Gibson PR, Collins J, Järnerot G, Hibi T, Rutgeerts P; PURSUIT-SC Study Group. Subcutaneous golimumab induces clinical response and remission in patients with moderate-to-severe ulcerative colitis. *Gastroenterology* 2014; **146**: 85-95; quiz e14-15 [PMID: 23735746 DOI: 10.1053/j.gastro.2013.05.048]

56 **Sandborn WJ**, Feagan BG, Marano C, Zhang H, Strauss R, Johanns J, Adedokun OJ, Guzzo C, Colombel JF, Reinisch W, Gibson PR, Collins J, Järnerot G, Rutgeerts P; PURSUIT-Maintenance Study Group. Subcutaneous golimumab maintains clinical response in patients with moderate-to-severe ulcerative colitis. *Gastroenterology* 2014; **146**: 96-109.e1 [PMID: 23770005 DOI: 10.1053/j.gastro.2013.06.010]

57 **Taxonera C**, Rodríguez C, Bertoletti F, Menchén L, Arribas J, Sierra M, Arias L, Martínez-Montiel P, Juan A, Iglesias E, Algaba A, Manceñido N, Rivero M, Barreiro-de Acosta M, López-Serrano P, Argüelles-Arias F, Gutierrez A, Busquets D, Gisbert JP, Olivares D, Calvo M, Alba C; Collaborators. Clinical Outcomes of Golimumab as First, Second or Third Anti-TNF Agent in Patients with Moderate-to-Severe Ulcerative Colitis. *Inflamm Bowel Dis* 2017; **23**: 1394-1402 [PMID: 28671873 DOI: 10.1097/MIB.0000000000001144]

58 **Feagan BG**, Rutgeerts P, Sands BE, Hanauer S, Colombel JF, Sandborn WJ, Van Assche G, Axler J, Kim HJ, Danese S, Fox I, Milch C, Sankoh S, Wyant T, Xu J, Parikh A; GEMINI 1 Study Group. Vedolizumab as induction and maintenance therapy for ulcerative colitis. *N Engl J Med* 2013; **369**: 699-710 [PMID: 23964932 DOI: 10.1056/NEJMoa1215734]

59 **Sandborn WJ**, Su C, Sands BE, D'Haens GR, Vermeire S, Schreiber S, Danese S, Feagan BG, Reinisch W, Niezychowski W, Friedman G, Lawendy N, Yu D, Woodworth D, Mukherjee A, Zhang H, Healey P, Panés J; OCTAVE Induction 1, OCTAVE Induction 2, and OCTAVE Sustain Investigators. Tofacitinib as Induction and Maintenance Therapy for Ulcerative Colitis. *N Engl J Med* 2017; **376**: 1723-1736 [PMID: 28467869 DOI: 10.1056/NEJMoa1606910]

60 **Pfizer.** Pfizer Announces Favorable Outcome of FDA Advisory Committee Meeting on Xeljanz® (Tofacitinib) for Moderately to Severely Active Ulcerative Colitis. Accessed March 28, 2018 Available from: URL: https://www.pfizer.com/news/press-release/press-release-detail/pfizer_announces_favorable_outcome_of_fda_advisory_committee_meeting_on_xeljanz_tofacitinib_for_moderately_to_severely_active_ulcerative_colitis

61 **Food and Drug Administration**. Guidance for industry: quality considerations in demonstrating biosimilarity to a therapeutic protein product to a reference product. Accessed March 28, 2018 Available from: URL: https://www.fda.gov/downloads/drugs/guidances/ucm291134.pdf

62 **Health Canada**. Patented Medicine Prices Review Board. Market Intelligence Report: Biologic Response Modifier Agents. Accessed March 28, 2018 Available from: URL: http://www.pmprb-cepmb.gc.ca/CMFiles/NPDUIS/PMBRB_16-240_MarketIntelligenceReport_E.pdf

63 **Danese S**, Bonovas S, Peyrin-Biroulet L. Biosimilars in IBD: from theory to practice. *Nat Rev Gastroenterol Hepatol* 2017; **14**: 22-31 [PMID: 27729659 DOI: 10.1038/nrgastro.2016.155]

64 **Park W**, Hrycaj P, Jeka S, Kovalenko V, Lysenko G, Miranda P, Mikazane H, Gutierrez-Ureña S, Lim M, Lee YA, Lee SJ, Kim H, Yoo DH, Braun J. A randomised, double-blind, multicentre, parallel-group, prospective study comparing the pharmacokinetics, safety, and efficacy of CT-P13 and innovator infliximab in patients with ankylosing spondylitis: the PLANETAS study. *Ann Rheum Dis* 2013; **72**: 1605-1612 [PMID: 23687259 DOI: 10.1136/annrheumdis-2012-203091]

65 **Yoo DH**, Hrycaj P, Miranda P, Ramiterre E, Piotrowski M, Shevchuk S, Kovalenko V, Prodanovic N, Abello-Banfi M, Gutierrez-Ureña S, Morales-Olazabal L, Tee M, Jimenez R, Zamani O, Lee SJ, Kim H, Park W, Müller-Ladner U. A randomised, double-blind, parallel-group study to demonstrate equivalence in efficacy and safety of CT-P13 compared with innovator infliximab when coadministered with methotrexate in patients with active rheumatoid arthritis: the PLANETRA study. *Ann Rheum Dis* 2013; **72**: 1613-1620 [PMID: 23687260 DOI: 10.1136/annrheumdis-2012-203090]

66 **Murphy C**, Sugrue K, Mohamad G, McCarthy J, Buckley M. Biosimilar but not the same. *J Crohns Colitis* 2015; **9**: S331-S332 [DOI: 10.1093/ecco-jcc/jju027.623]

67 **Jung YS**, Park DI, Kim YH, Lee JH, Seo PJ, Cheon JH, Kang HW, Kim JW. Efficacy and safety of CT-P13, a biosimilar of infliximab, in patients with inflammatory bowel disease: A retrospective multicenter study. *J Gastroenterol Hepatol* 2015; **30**: 1705-1712 [PMID: 25974251 DOI: 10.1111/jgh.12997]

68 **Kang YS**, Moon HH, Lee SE, Lim YJ, Kang HW. Clinical Experience of the Use of CT-P13, a Biosimilar to Infliximab in Patients with Inflammatory Bowel Disease: A Case Series. *Dig Dis Sci* 2015; **60**: 951-956 [PMID: 25326115 DOI: 10.1007/s10620-014-3392-z]

69 **Gecse KB**, Lovász BD, Farkas K, Banai J, Bene L, Gasztonyi B, Golovics PA, Kristóf T, Lakatos L, Csontos ÁA, Juhász M, Nagy F, Palatka K, Papp M, Patai Á, Lakner L, Salamon Á, Szamosi T, Szepes Z, Tóth GT, Vincze Á, Szalay B, Molnár T, Lakatos PL. Efficacy and Safety of the Biosimilar Infliximab CT-P13 Treatment in Inflammatory Bowel Diseases: A Prospective, Multicentre, Nationwide Cohort. *J Crohns Colitis* 2016; **10**: 133-140 [PMID: 26661272 DOI: 10.1093/ecco-jcc/jjv220]

70 **Fiorino G**, Manetti N, Armuzzi A, Orlando A, Variola A, Bonovas S, Bossa F, Maconi G, D'Incà R, Lionetti P, Cantoro L, Fries W, Annunziata ML, Costa F, Terpin MM, Biancone L, Cortelezzi CC, Amato A, Ardizzone S, Danese S, Guidi L, Rizzuto G, Massella A, Andriulli A, Massari A, Lorenzon G, Ghione S, Kohn A, Ventra A, Annese V; PROSIT-BIO Cohort. The PROSIT-BIO Cohort: A Prospective Observational Study of Patients with Inflammatory Bowel Disease Treated with Infliximab Biosimilar. *Inflamm Bowel Dis* 2017; **23**: 233-243 [PMID: 28092307 DOI: 10.1097/MIB.0000000000000995]

71 **Danese S**, Fiorino G, Raine T, Ferrante M, Kemp K, Kierkus J, Lakatos PL, Mantzaris G, van der Woude J, Panes J, Peyrin-Biroulet L. ECCO Position Statement on the Use of Biosimilars for Inflammatory Bowel Disease-An Update. *J Crohns Colitis* 2017; **11**: 26-34 [PMID: 27927718 DOI: 10.1093/ecco-jcc/jjw198]

72 **Kim YH,** Ye BD, Pesegova M, Alexeeva O, Osipenko M, Lahat

A, Dorofeyev A, Salamon A, Fishman S, Levchenko O, Cheon JH, Scribano ML, Mateescu RB, Lee KM, Eun CS, Lee SJ, Lee SY, Kudrin A. Phase Ⅲ Randomized Controlled Trial to Compare Biosimilar Infliximab (Ct-P13) With Innovator Infliximab in Patients With Active Crohn's Disease: 1-Year Maintenance and Switching Results. *United European Gastroenterol J* 2017; **5**: Abstract

73   **Jørgensen KK**, Olsen IC, Goll GL, Lorentzen M, Bolstad N, Haavardsholm EA, Lundin KEA, Mørk C, Jahnsen J, Kvien TK; NOR-SWITCH study group. Switching from originator infliximab to biosimilar CT-P13 compared with maintained treatment with originator infliximab (NOR-SWITCH): a 52-week, randomised, double-blind, non-inferiority trial. *Lancet* 2017; **389**: 2304-2316 [PMID: 28502609 DOI: 10.1016/S0140-6736(17)30068-5]

74   **Ben-Horin S**, Yavzori M, Benhar I, Fudim E, Picard O, Ungar B, Lee S, Kim S, Eliakim R, Chowers Y. Cross-immunogenicity: antibodies to infliximab in Remicade-treated patients with IBD similarly recognise the biosimilar Remsima. *Gut* 2016; **65**: 1132-1138 [PMID: 25897019 DOI: 10.1136/gutjnl-2015-309290]

75   **Gils A**, Van Stappen T, Dreesen E, Storme R, Vermeire S, Declerck PJ. Harmonization of Infliximab and Anti-Infliximab Assays Facilitates the Comparison Between Originators and Biosimilars in Clinical Samples. *Inflamm Bowel Dis* 2016; **22**: 969-975 [PMID: 26954707 DOI: 10.1097/MIB.0000000000000709]

76   **Mao EJ**, Hazlewood GS, Kaplan GG, Peyrin-Biroulet L, Ananthakrishnan AN. Systematic review with meta-analysis: comparative efficacy of immunosuppressants and biologics for reducing hospitalisation and surgery in Crohn's disease and ulcerative colitis. *Aliment Pharmacol Ther* 2017; **45**: 3-13 [PMID: 27862107 DOI: 10.1111/apt.13847]

77   **Danese S**, Vuitton L, Peyrin-Biroulet L. Biologic agents for IBD: practical insights. *Nat Rev Gastroenterol Hepatol* 2015; **12**: 537-545 [PMID: 26284562 DOI: 10.1038/nrgastro.2015.135]

78   **Lichtenstein GR**, Feagan BG, Cohen RD, Salzberg BA, Diamond RH, Price S, Langholff W, Londhe A, Sandborn WJ. Serious infection and mortality in patients with Crohn's disease: more than 5 years of follow-up in the TREAT™ registry. *Am J Gastroenterol* 2012; **107**: 1409-1422 [PMID: 22890223 DOI: 10.1038/ajg.2012.218]

79   **Hazlewood GS**, Rezaie A, Borman M, Panaccione R, Ghosh S, Seow CH, Kuenzig E, Tomlinson G, Siegel CA, Melmed GY, Kaplan GG. Comparative effectiveness of immunosuppressants and biologics for inducing and maintaining remission in Crohn's disease: a network meta-analysis. *Gastroenterology* 2015; **148**: 344-54.e5; quiz e14-5 [PMID: 25448924 DOI: 10.1053/j.gastro.2014.10.011]

80   **Stidham RW**, Lee TC, Higgins PD, Deshpande AR, Sussman DA, Singal AG, Elmunzer BJ, Saini SD, Vijan S, Waljee AK. Systematic review with network meta-analysis: the efficacy of anti-TNF agents for the treatment of Crohn's disease. *Aliment Pharmacol Ther* 2014; **39**: 1349-1362 [PMID: 24749763 DOI: 10.1111/apt.12749]

81   **Danese S**, Fiorino G, Peyrin-Biroulet L, Lucenteforte E, Virgili G, Moja L, Bonovas S. Biological agents for moderately to severely active ulcerative colitis: a systematic review and network meta-analysis. *Ann Intern Med* 2014; **160**: 704-711 [PMID: 24842416 DOI: 10.7326/M13-2403]

82   **AbbVie Inc.** Study to Evaluate Efficacy and Safety of Two Drug Regimens in Subjects With Moderate to Severe Crohn's Disease. [accessed 2018 Mar 28]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT02065570 ClinicalTrials.gov Identifier: NCT02065570

83   **AbbVie Inc.** Study to Evaluate the Safety and Efficacy of Two Drug Regimens in Subjects With Moderate to Severe Ulcerative Colitis. [accessed 2018 Mar 28]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT02065622 ClinicalTrials.gov Identifier: NCT02065622

84   **Järnerot G**, Hertervig E, Friis-Liby I, Blomquist L, Karlén P, Grännö C, Vilien M, Ström M, Danielsson A, Verbaan H, Hellström PM, Magnuson A, Curman B. Infliximab as rescue therapy in severe to moderately severe ulcerative colitis: a randomized, placebo-controlled study. *Gastroenterology* 2005; **128**: 1805-1811 [PMID: 15940615 DOI: 10.1053/j.gastro.2005.03.003]

85   **Colombel JF**, Schwartz DA, Sandborn WJ, Kamm MA, D'Haens G, Rutgeerts P, Enns R, Panaccione R, Schreiber S, Li J, Kent JD, Lomax KG, Pollack PF. Adalimumab for the treatment of fistulas in patients with Crohn's disease. *Gut* 2009; **58**: 940-948 [PMID: 19201775 DOI: 10.1136/gut.2008.159251]

86   **Dreesen E**, Bossuyt P, Mulleman D, Gils A, Pascual-Salcedo D. Practical recommendations for the use of therapeutic drug monitoring of biopharmaceuticals in inflammatory diseases. *Clin Pharmacol* 2017; **9**: 101-111 [PMID: 29042821 DOI: 10.2147/CPAA.S138414]

87   **Vande Casteele N**, Ferrante M, Van Assche G, Ballet V, Compernolle G, Van Steen K, Simoens S, Rutgeerts P, Gils A, Vermeire S. Trough concentrations of infliximab guide dosing for patients with inflammatory bowel disease. *Gastroenterology* 2015; **148**: 1320-1329. e3 [PMID: 25724455 DOI: 10.1053/j.gastro.2015.02.031]

88   **Vande Casteele N,** Compernolle G, Ballet V, Van Assche G, Gils A, Vermeire S, Rutgeerts P. OP11 Individualised infliximab treatment using therapeutic drug monitoring: A prospective controlled Trough level Adapted infliXImab Treatment (TAXIT) trial. *J Crohns Colitis* 2012; **6**: S6 [DOI: 10.1016/S1873-9946(12)60012-9]

89   **D'Haens G,** Vermeire S, Lambrecht G, Baert F, Bossuyt P, Nachury M, Buisson A, Bouhnik Y, Filippi J, vande Woude J, Van Hootegem P, Moreau J, Louis E, Franchimont D, De Vos M, Mana F, Peyrin-Biroulet L, Brixi H, Allez M, Caenepeel P, Aubourg A, Oldenburg B, Pierik M, Gils A, Chevret S, Laharie D. OP029 Drug-concentration versus symptom-driven dose adaptation of Infliximab in patients with active Crohn's disease: a prospective, randomised, multicentre trial (Tailorix). *J Crohns Colitis* 2016; **10**: S24.1-S24 [DOI: 10.1093/ecco-jcc/jjw019.028]

90   **Panaccione R**, Ghosh S, Middleton S, Márquez JR, Scott BB, Flint L, van Hoogstraten HJ, Chen AC, Zheng H, Danese S, Rutgeerts P. Combination therapy with infliximab and azathioprine is superior to monotherapy with either agent in ulcerative colitis. *Gastroenterology* 2014; **146**: 392-400.e3 [PMID: 24512909 DOI: 10.1053/j.gastro.2013.10.052]

91   **Matsumoto T**, Motoya S, Watanabe K, Hisamatsu T, Nakase H, Yoshimura N, Ishida T, Kato S, Nakagawa T, Esaki M, Nagahori M, Matsui T, Naito Y, Kanai T, Suzuki Y, Nojima M, Watanabe M, Hibi T; DIAMOND study group. Adalimumab Monotherapy and a Combination with Azathioprine for Crohn's Disease: A Prospective, Randomized Trial. *J Crohns Colitis* 2016; **10**: 1259-1266 [PMID: 27566367 DOI: 10.1093/ecco-jcc/jjw152]

92   **Andersen NN**, Jess T. Risk of infections associated with biological treatment in inflammatory bowel disease. *World J Gastroenterol* 2014; **20**: 16014-16019 [PMID: 25473153 DOI: 10.3748/wjg.v20.i43.16014]

93   **Magro F**, Peyrin-Biroulet L, Sokol H, Aldeger X, Costa A, Higgins PD, Joyce JC, Katsanos KH, Lopez A, de Xaxars TM, Toader E, Beaugerie L. Extra-intestinal malignancies in inflammatory bowel disease: results of the 3rd ECCO Pathogenesis Scientific Workshop (Ⅲ). *J Crohns Colitis* 2014; **8**: 31-44 [PMID: 23721759 DOI: 10.1016/j.crohns.2013.04.006]

94   **Drobne D**, Bossuyt P, Breynaert C, Cattaert T, Vande Casteele N, Compernolle G, Jürgens M, Ferrante M, Ballet V, Wollants WJ, Cleynen I, Van Steen K, Gils A, Rutgeerts P, Vermeire S, Van Assche G. Withdrawal of immunomodulators after co-treatment does not reduce trough level of infliximab in patients with Crohn's disease. *Clin Gastroenterol Hepatol* 2015; **13**: 514-521.e4 [PMID: 25066841 DOI: 10.1016/j.cgh.2014.07.027]

95   **Van Assche G**, Magdelaine-Beuzelin C, D'Haens G, Baert F, Noman M, Vermeire S, Ternant D, Watier H, Paintaud G, Rutgeerts P. Withdrawal of immunosuppression in Crohn's disease treated with scheduled infliximab maintenance: a randomized trial. *Gastroenterology* 2008; **134**: 1861-1868 [PMID: 18440315 DOI: 10.1053/j.gastro.2008.03.004]

96   **Takeda.** Vedolizumab IV 300 mg in the Treatment of Fistulizing Crohn's Disease (ENTERPRISE). [accessed 2018 Mar 28]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National

Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT02630966 ClinicalTrials.gov Identifier: NCT02630966

97   **D'Haens G**. An Open Label Phase 4 Study to Evaluate Efficacy of Early Versus Late Use of Vedolizumab in Ulcerative Colitis (LOVE-UC). [accessed 2018 Mar 28]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT02646657 ClinicalTrials.gov Identifier: NCT02646657

98   **D'Haens G**. A Study to Evaluate Efficacy, of Early Versus Late Use of Vedolizumab in Crohn's Disease: the LOVE-CD Study (LOVE-CD). [accessed 2018 Mar 28]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT02646683 ClinicalTrials.gov Identifier: NCT02646683

99   **Takeda.** Triple Combination Therapy in High Risk Crohn's Disease. [accessed 2018 Mar 28]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT02764762 ClinicalTrials.gov Identifier: NCT02764762

100   **Jess T**, Riis L, Vind I, Winther KV, Borg S, Binder V, Langholz E, Thomsen OØ, Munkholm P. Changes in clinical characteristics, course, and prognosis of inflammatory bowel disease during the last 5 decades: a population-based study from Copenhagen, Denmark. *Inflamm Bowel Dis* 2007; **13**: 481-489 [PMID: 17206705 DOI: 10.1002/ibd.20036]

101   **Solberg IC**, Vatn MH, Høie O, Stray N, Sauar J, Jahnsen J, Moum B, Lygren I; IBSEN Study Group. Clinical course in Crohn's disease: results of a Norwegian population-based ten-year follow-up study. *Clin Gastroenterol Hepatol* 2007; **5**: 1430-1438 [PMID: 18054751 DOI: 10.1016/j.cgh.2007.09.002]

102   **Lakatos PL**. Prediction of disease course in inflammatory bowel diseases. *World J Gastroenterol* 2010; **16**: 2589-2590 [PMID: 20518078 DOI: 10.3748/wjg.v16.i21.2589]

103   **Atreya R**, Neumann H, Neufert C, Waldner MJ, Billmeier U, Zopf Y, Willma M, App C, Münster T, Kessler H, Maas S, Gebhardt B, Heimke-Brinck R, Reuter E, Dörje F, Rau TT, Uter W, Wang TD, Kiesslich R, Vieth M, Hannappel E, Neurath MF. In vivo imaging using fluorescent antibodies to tumor necrosis factor predicts therapeutic response in Crohn's disease. *Nat Med* 2014; **20**: 313-318 [PMID: 24562382 DOI: 10.1038/nm.3462]

104   **Van den Brande JM**, Koehler TC, Zelinkova Z, Bennink RJ, te Velde AA, ten Cate FJ, van Deventer SJ, Peppelenbosch MP, Hommes DW. Prediction of antitumour necrosis factor clinical efficacy by real-time visualisation of apoptosis in patients with Crohn's disease. *Gut* 2007; **56**: 509-517 [PMID: 17082252 DOI: 10.1136/gut.2006.105379]

105   **D'Haens G**, Baert F, van Assche G, Caenepeel P, Vergauwe P, Tuynman H, De Vos M, van Deventer S, Stitt L, Donner A, Vermeire S, Van De Mierop FJ, Coche JR, van der Woude J, Ochsenkühn T, van Bodegraven AA, Van Hootegem PP, Lambrecht GL, Mana F, Rutgeerts P, Feagan BG, Hommes D; Belgian Inflammatory Bowel Disease Research Group; North-Holland Gut Club. Early combined immunosuppression or conventional management in patients with newly diagnosed Crohn's disease: an open randomised trial. *Lancet* 2008; **371**: 660-667 [PMID: 18295023 DOI: 10.1016/S0140-6736(08)60304-9]

106   **Khanna R**, Bressler B, Levesque BG, Zou G, Stitt LW, Greenberg GR, Panaccione R, Bitton A, Paré P, Vermeire S, D'Haens G, MacIntosh D, Sandborn WJ, Donner A, Vandervoort MK, Morris JC, Feagan BG; REACT Study Investigators. Early combined immunosuppression for the management of Crohn's disease (REACT): a cluster randomised controlled trial. *Lancet* 2015; **386**: 1825-1834 [PMID: 26342731 DOI: 10.1016/S0140-6736(15)00068-9]

107   **Höie O**, Wolters F, Riis L, Aamodt G, Solberg C, Bernklev T, Odes S, Mouzas IA, Beltrami M, Langholz E, Stockbrügger R, Vatn M, Moum B; European Collaborative Study Group of Inflammatory Bowel Disease (EC-IBD). Ulcerative colitis: patient characteristics may predict 10-yr disease recurrence in a European-wide population-based cohort. *Am J Gastroenterol* 2007; **102**: 1692-1701 [PMID:

17555460 DOI: 10.1111/j.1572-0241.2007.01265.x]

108   **Jess T**, Rungoe C, Peyrin-Biroulet L. Risk of colorectal cancer in patients with ulcerative colitis: a meta-analysis of population-based cohort studies. *Clin Gastroenterol Hepatol* 2012; **10**: 639-645 [PMID: 22289873 DOI: 10.1016/j.cgh.2012.01.010]

109   **Solberg IC**, Høivik ML, Cvancarova M, Moum B; IBSEN Study Group. Risk matrix model for prediction of colectomy in a population-based study of ulcerative colitis patients (the IBSEN study). *Scand J Gastroenterol* 2015; **50**: 1456-1462 [PMID: 26139389 DOI: 10.3109/00365521.2015.1064991]

110   **Janssen Research & Development, LLC.** A Study of Safety and Effectiveness of JNJ-54781532 in Patients With Moderately to Severely Active Ulcerative Colitis. [accessed 2018 Mar 28]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT01959282 ClinicalTrials.gov Identifier: NCT01959282

111   **Monteleone G**, Neurath MF, Ardizzone S, Di Sabatino A, Fantini MC, Castiglione F, Scribano ML, Armuzzi A, Caprioli F, Sturniolo GC, Rogai F, Vecchi M, Atreya R, Bossa F, Onali S, Fichera M, Corazza GR, Biancone L, Savarino V, Pica R, Orlando A, Pallone F. Mongersen, an oral SMAD7 antisense oligonucleotide, and Crohn's disease. *N Engl J Med* 2015; **372**: 1104-1113 [PMID: 25785968 DOI: 10.1056/NEJMoa1407250]

112   **Delgado A**, Martínez-Cartro M. Therapeutic Potential of the Modulation of Sphingosine-1-Phosphate Receptors. *Curr Med Chem* 2016; **23**: 242-264 [PMID: 26639095 DOI: 10.2174/0929867323666151207111509]

113   **Marafini I**, Zorzi F, Codazza S, Pallone F, Monteleone G. TGF-Beta signaling manipulation as potential therapy for IBD. *Curr Drug Targets* 2013; **14**: 1400-1404 [PMID: 23489130 DOI: 10.2174/13894501113149990157]

114   **Valles-Colomer M**, Darzi Y, Vieira-Silva S, Falony G, Raes J, Joossens M. Meta-omics in Inflammatory Bowel Disease Research: Applications, Challenges, and Guidelines. *J Crohns Colitis* 2016; **10**: 735-746 [PMID: 26802086 DOI: 10.1093/ecco-jcc/jjw024]

115   **Louis E**, El Ghoul Z, Vermeire S, Dall'Ozzo S, Rutgeerts P, Paintaud G, Belaiche J, De Vos M, Van Gossum A, Colombel JF, Watier H. Association between polymorphism in IgG Fc receptor IIIa coding gene and biological response to infliximab in Crohn's disease. *Aliment Pharmacol Ther* 2004; **19**: 511-519 [PMID: 14987319 DOI: 10.1111/j.1365-2036.2004.01871.x]

116   **Urcelay E**, Mendoza JL, Martinez A, Fernandez L, Taxonera C, Diaz-Rubio M, de la Concha EG. IBD5 polymorphisms in inflammatory bowel disease: association with response to infliximab. *World J Gastroenterol* 2005; **11**: 1187-1192 [PMID: 15754402 DOI: 10.3748/wjg.v11.i8.1187]

117   **Harbord M**, Eliakim R, Bettenworth D, Karmiris K, Katsanos K, Kopylov U, Kucharzik T, Molnár T, Raine T, Sebastian S, de Sousa HT, Dignass A, Carbonnel F; European Crohn's and Colitis Organisation [ECCO]. Third European Evidence-based Consensus on Diagnosis and Management of Ulcerative Colitis. Part 2: Current Management. *J Crohns Colitis* 2017; **11**: 769-784 [PMID: 28513805 DOI: 10.1093/ecco-jcc/jjx009]

118   **Hindryckx P**, Vande Casteele N, Novak G, Khanna R, D'Haens G, Sandborn WJ, Danese S, Jairath V, Feagan BG. The Expanding Therapeutic Armamentarium for Inflammatory Bowel Disease: How to Choose the Right Drug[s] for Our Patients? *J Crohns Colitis* 2018; **12**: 105-119 [PMID: 28961959 DOI: 10.1093/ecco-jcc/jjx117]

119   **Gottlieb AB**. Infliximab for psoriasis. *J Am Acad Dermatol* 2003; **49**: S112-S117 [PMID: 12894134 DOI: 10.1016/S0190-9622(03)01143-5]

120   **Alwawi EA**, Mehlis SL, Gordon KB. Treating psoriasis with adalimumab. *Ther Clin Risk Manag* 2008; **4**: 345-351 [PMID: 18728850 DOI: 10.2147/TCRM.S1265]

121   **Mounach A**, El Maghraoui A. Efficacy and safety of adalimumab in ankylosing spondylitis. *Open Access Rheumatol* 2014; **6**: 83-90 [PMID: 27790037 DOI: 10.2147/OARRR.S44550]

122   **Dattola A**, Cannizzaro MV, Mazzeo M, Bianchi L. Certolizumab Pegol in the Treatment of Psoriasis and Psoriatic Arthritis: Preliminary Real-Life Data. *Dermatol Ther* (Heidelb) 2017; **7**: 485-492 [PMID: 29139035 DOI: 10.1007/s13555-017-0208-z]

123   **Landewé R**, Braun J, Deodhar A, Dougados M, Maksymowych WP, Mease PJ, Reveille JD, Rudwaleit M, van der Heijde D, Stach C, Hoepken B, Fichtner A, Coteur G, de Longueville M, Sieper J. Efficacy of certolizumab pegol on signs and symptoms of axial spondyloarthritis including ankylosing spondylitis: 24-week results of a double-blind randomised placebo-controlled Phase 3 study. *Ann Rheum Dis* 2014; **73**: 39-47 [PMID: 24013647 DOI: 10.1136/annrheumdis-2013-204231]

124   **Yang H**, Epstein D, Bojke L, Craig D, Light K, Bruce I, Sculpher M, Woolacott N. Golimumab for the treatment of psoriatic arthritis.

Health Technol Assess 2011; **15** Suppl 1: 87-95 [PMID: 21609657 DOI: 10.3310/hta15suppl1/10]

125   **Poddubnyy D**, Hermann KG, Callhoff J, Listing J, Sieper J. Ustekinumab for the treatment of patients with active ankylosing spondylitis: results of a 28-week, prospective, open-label, proof-of-concept study (TOPAS). *Ann Rheum Dis* 2014; **73**: 817-823 [PMID: 24389297 DOI: 10.1136/annrheumdis-2013-204248]

126   **Savage LJ**, Wittmann M, McGonagle D, Helliwell PS. Ustekinumab in the Treatment of Psoriasis and Psoriatic Arthritis. *Rheumatol Ther* 2015; **2**: 1-16 [PMID: 27747495 DOI: 10.1007/s40744-015-0010-2]

Prognostic significance of tumor immune microenvironment and immunotherapy: Novel insights and future perspectives in gastric cancer

Daniela Cornelia Lazăr, Mihaela Flavia Avram, Ioan Romoșan, Mărioara Cornianu, Sorina Tăban, Adrian Goldiș

Daniela Cornelia Lazăr, Ioan Romoșan, Department of Internal Medicine I, University Medical Clinic, University of Medicine and Pharmacy "Victor Babeş", Timişoara 300041, Timiş County, Romania

Mihaela Flavia Avram, Department of Surgery X, 1st Surgery Clinic, University of Medicine and Pharmacy "Victor Babeş", Timişoara 300041, Timiş County, Romania

Mărioara Cornianu, Sorina Tăban, Department of Pathology, University of Medicine and Pharmacy "Victor Babeş", Timişoara 300041, Timiş County, Romania

Adrian Goldiş, Department of Gastroenterology and Hepatology, University of Medicine and Pharmacy "Victor Babeş", Timişoara 300041, Timiş County, Romania

ORCID number: Daniela Cornelia Lazăr (0000-0002-6984-0046); Mihaela Flavia Avram (0000-0001-8582-521x); Ioan Romoșan (0000-0003-4883-5069); Mărioara Cornianu (0000-0001-5675-5339); Sorina Tăban (0000-0002-3971-2756); Adrian Goldiş (0000-0003-0670-8862).

Author contributions: All the authors contributed equally to this work.

Conflict-of-interest statement: The authors have no conflict of interest to report.

Manuscript source: Invited manuscript

Correspondence to: Daniela Cornelia Lazăr, MD, PhD, Lecturer, Department of Internal Medicine I, University of Medicine and Pharmacy "Victor Babeş", Piata Eftimie Murgu No.2, Timişoara 300041, Timiş County, Romania. lazar.daniela@umft.ro
Telephone: +4-256-220484
Fax: +4-256-220484

Abstract

Despite a decrease in gastric cancer incidence, the development of novel biologic agents and combined therapeutic strategies, the prognosis of gastric cancer remains poor. Recently, the introduction of modern immunotherapy, especially using immune checkpoint inhibitors, led to an improved prognosis in many cancers. The use of immunotherapy was also associated with manageable adverse event profiles and promising results in the treatment of patients with gastric cancer, especially in heavily pretreated patients. These data have led to an accelerated approval of some checkpoint inhibitors in this setting. Understanding the complex relationship between the host immune microenvironment and tumor and the immune escape phenomenon leading to cancer occurrence and progression will subsequently lead to the identification of prognostic immune markers. Furthermore, this understanding will result in the discovery of both new mechanisms for blocking tumor immunosuppressive signals and pathways to stimulate the local immune response by targeting and modulating different subsets of immune cells. Due to the molecular heterogeneity of gastric cancers associated with different

clinico-biologic parameters, immune markers expression and prognosis, novel immunotherapy algorithms should be personalized and addressed to selected subsets of gastric tumors, which have been proven to elicit the best clinical responses. Future perspectives in the treatment of gastric cancer include tailored dual immunotherapies or a combination of immunotherapy with other targeted agents with synergistic antitumor effects.

Key words: Immunotherapy; Prognostic significance; Tumor immune microenvironment; Immune checkpoint inhibitors; Gastric cancer

Core tip: The use of modern immunotherapy, including adoptive cell therapy, vaccines, and especially immune therapy using checkpoint inhibitors, has led to encouraging results in clinical trials including gastric cancer patients. This review analyzes the relationship between immune microenvironment profile of the host and tumor development, identification of the immune prognostic markers and future perspectives of immunotherapeutic strategies. The treatment algorithm should be adapted to the specific molecular profile of the gastric cancer subtype in order to obtain maximum clinical benefits.

INTRODUCTION

Early estimates situated gastric cancer in the first place as being the most frequent neoplasia (1975). Despite a decrease in gastric cancer incidents during the last decades, it remains a major health problem globally, with almost one million new cases diagnosed in 2012 (6.8% of the total), after tumors of the lung, breast, colorectum and prostate. There is a high geographical variation in gastric cancer incidence. Approximately 70% of cases occur in developing countries; half of the total number occurs in Eastern Asia, especially China; the incidence rates are approximately twice in men *vs* women. Overall, this type of tumor represents the third leading cause of cancer death in both sexes, accounting for 723,000 deaths in 2012 (8.8% of the total number of cases). The highest mortality rates are seen in Eastern Asia, whereas the lowest rates occur in Northern America; also, high mortality rates are encountered in Central and Eastern Europe and in Central and South America, respectively[1].

Most gastric cancers are diagnosed at an advanced stage, whereas another 25%-50% of cases will develop metastases during the outcome of the disease. Although surgical resection remains the main treatment with curative-intent in gastric cancer patients, there is a poor associated 5-year survival rate of approximately 20%-25%. Therefore, additional treatments (neo-adjuvant/adjuvant), such as chemotherapy and radiotherapy where associated with tumor resection, unfortunately lead to only modest survival benefits. In advanced stages, approximately 50% of cases present local/systemic recurrence after adjuvant treatment, and only 10%-15% of cases achieve a 5-year overall survival[2]. In the metastatic stage, the backbone of treatment is represented by palliative chemotherapy, associated with a poor median overall survival, of approximately 8-10 mo[3]. Despite recent advances using novel biologic therapeutic agents, with the exception of trastuzumab [anti-human growth factor receptor 2 (HER2) monoclonal antibody] and ramucirumab [fully humanized monoclonal antibody receptor antagonist to bind vascular endothelial growth factor receptor 2 (VEGFR-2)], showing beneficial results by improving overall survival (OS), and therefore approved in first-line (in association with standard chemotherapeutic regimens) and second-line settings, respectively (as monotherapy, or in association with chemotherapy), in advanced and metastatic gastric cancers, clinical trials assessing other targeted agents showed disappointing results in gastric cancer[4-6].

Recently, the therapeutic algorithm and prognosis of many tumors changed radically by introducing immunotherapy, especially using immune checkpoint inhibitors, and the first drug of this class approved by the United States Food and Drug Administration (FDA) was ipilimumab, an anticytotoxic T lymphocyte antigen-4 (CTLA-4) antibody, used in the treatment of advanced melanoma (2011)[7,8]. Afterwards, immune checkpoint inhibitors, which are antagonists of the programmed death (PD)-1/PD-ligand 1 (PD-L1) pathway, were approved by the FDA for the treatment of different tumors, such as melanoma, non-small cell lung cancer (NSCLC), urothelial/renal cell carcinoma, squamous cell carcinoma of the head and neck, Merkel cell carcinoma and Hodgkin's lymphoma[9].

MOLECULAR CLASSIFICATION OF GASTRIC CANCER

The following main histological classifications of gastric cancer have routinely been used: the World Health Organization (WHO) classification[10] that categorizes four histological subtypes, namely, papillary, tubular, mucinous and poorly cohesive, and Lauren's classification, dividing gastric cancers into intestinal, diffuse and mixed type[11].

Because these two classifications are not able to direct specific therapeutic strategies and, additionally, because the group of gastric cancers includes heterogeneity of tumors, there was a need to elaborate new classifications capable of stratifying patients regarding tumor behavior, prognosis and response to specific treatments. For the first time, the molecular assessment of gastric cancer

patients was proven to add benefits in the context of the TOGA trial in which a combined treatment with classical chemotherapy and trastuzumab showed an improvement of survival in the subgroup of patients overexpressing HER2[4]. Moreover, the behavior of the tumor and the outcome proved to be different in cases of Asian patients *vs* Caucasians included in several clinical trials[12].

In 2013, Singapore researchers identified three different molecular subtypes of gastric cancer: proliferative (high genomic instability, TP53 mutation), metabolic (high response to 5-FU chemotherapy), and mesenchymal (stem cell-like cancers that are sensitive to PIK3CA-mTOR inhibitors)[13].

The aim of "The Cancer Genome Atlas (TCGA)" project (2014) was to develop a new molecular classification of gastric cancer with clinical impact and to identify the main dysregulated pathways of each subtype of gastric tumors. The TGCA research group divided gastric cancer into four genomic subtypes:

Chromosomal instability (CIN): Includes approximately 50% of cases, most of the tumors are located at the gastro-esophageal junction or cardia[14]; leads to a loss or gain of some oncogenes and tumor suppressor genes[15]; has a high frequency of TP53 gene and receptor tyrosine kinase mutations and amplifications of cell cycle genes[16]; and has amplifications in oncogene pathways (HER2, BRAF, EGFR, MET, and RAS)[17].

Microsatellite instability: Tumor testing methodologies include immunohistochemistry for abnormal absence of MMR protein expression or polymerase chain reaction (PCR) for Microsatellite instability analysis (MSI), to evaluate for MSI-H on a tumor specimen. Immunohistochemistry can predict which gene is most likely to be mutated, the gene for the affected protein. Interpretation of immunohistochemical reports can sometimes be confusing, as "positive" should mean the abnormal absence of MMR protein expression, in contrast to normal presence of expression. MSI testing panels may consist of mononucleotide and dincleotid markers. For classifying MSI, a panel of five markers for the analysis of MSI was recommended by the National Cancer Institute, including two mononucleotide and two dinucleotide repeats. In the case that ≥ 2 of the five markers show instability, the genotypes are grouped into high-frequency (MSI-H); when only one marker shows instability, into low-frequency (MSI-L), and when no marker shows instability, into microsatellite stable (MSS). MSI-H consists of 30%-40% instability markers, while MSI-L of < 30%-40%. Bethesda Guidelines have stated that MSI-H can be defined when instability is present at mononucleotide loci and MSI-L when instability is limited at only dinucleotide loci; mononucleotide repeats were demonstrated to be more sensitive *vs* dinucleotide loci in detecting MSI. Some studies have shown that both immunohistochemistry and MSI are cost-effective and useful for selecting high-risk patients. A review showed that the sensitivities

of MSI and immunohistochemical testing are 77% to 89%, and 83%, respectively; specificities are 90% and 89%, respectively. Some patients may have MSI or abnormal immunohistochemistry due to sporadic development of cancer, rather than an underlying genetic (germline) mutation. MSI accounts for 15%-30% of gastric cancers; most often includes tumors of intestinal type, antral location, females and older patients[18,19]; shows mutations in DNA mismatch repair genes, such as MLH1 or MLH2, that lead to the dysfunction of DNA mismatch repair enzymes[20]; is reported in a meta-analysis that demonstrated a 37% reduced mortality risk and prolonged OS in gastric cancer patients with MSI-high (MSI-H) *vs* MSI-low (MSI-L)[21]; is associated in non-colorectal cancer with an increased frequency of somatic mutation and amplification of tumor antigens; therefore, an increased sensitivity to treatment with PD-1 immune checkpoint inhibitors[22,23]; has increased intratumor infiltrate[24]; is reported in studies that show an increased activity of pembrolizumab in gastric cancer[25] and assess the efficacy of nivolumab ± ipilimumab in MSI-H gastrointestinal cancers[26]; and is possibly being considered in the development of a preventive vaccine using neopeptides affecting MSI carcinogenesis[27].

Genomic stability: Accounts for approximately 20% of gastric cancer cases; has an increased frequency of the diffuse type; indicates main somatic genomic alterations: CDH1, ARIDIA, RHOA; and presents a recurrent inter-chromosomal translocation involved in cellular motility[28].

Epstein Barr virus-associated: Epstein Barr virus (EBV) is detected by *in situ* hybridization or PCR in approximately 10% of gastric cancer patients[29]; it seems to increase ten times the gastric cancer risk, especially in Far East Asian patients[30], and is more frequent in younger patients[29]; it has a better response to immunotherapy and better survival[31]; it shows that the PD-L1 gene is frequently amplified; approximately 15% of EBV+ tumors present amplification of chromosomal region 9p24.1 (locus of PD-L1, PD-L2)[32]; EBV+ is found in approximately 50% of tumor cells and 94% of immune cells; therefore, PD-1 testing could predict a response to immunotherapy in this subset of patients[12]; and it shows PIK3CA mutations that could be targeted using PI(3)-kinase inhibition, DNA hypermethylation and JAK2 mutations[28].

Taking into account specific characteristics of the four subtypes of gastric cancer, it was highlighted that EBV-associated and MSI categories are associated with the best responses to immune therapeutic strategies.

The Asian Cancer Research Group (ACRG) proposed a molecular classification of four molecular subtypes for gastric cancer (2015): one subtype was related to the epithelial-to-mesenchymal transition (MSS/EMT) phenotype, which was associated with highest recurrence frequency, and another subtype was related to the phenotype of MSI, cytokine signaling, cell proliferation and methylation signals, including hypermutated tumors,

which was associated with the best overall prognosis. The remaining of the non-EMT and non-MSI patients were further divided into MSS/p53- and MSS/p53+ molecular subtypes and were associated with an intermediate overall prognosis and recurrence[33,34].

Comparing TCGA *vs* ACRG subtypes of gastric cancers, one may notice a resemblance between MSI tumors, GS and MSS/EMT subtypes, EBV and MSS/TP53+ subtypes, and CIN and MSS/TP53- tumors, respectively[13].

The development of genotyping different subtypes of gastric cancer will provide a guide to molecular targeted drugs that should be investigated in large clinical trials on specific subsets of gastric tumor patients in the future.

HOST IMMUNE RESPONSE IN GASTRIC CANCER PATIENTS

Antitumor immune response of the host
The term "immunosurveillance" refers to the capacity of the host immune system to identify the tumor cells as "non self", and subsequently to kill them[35]. The immune response includes both innate immunity (represented by macrophages, dendritic cells, and natural killer cells), and specific adaptive immunity (T and B lymphocytes).

The host protective response and the capacity of the tumor to surpass the host immune response are defined as "cancer immunoediting", which is a process comprising three progressive steps:

Elimination phase: In this stage, natural killer (NK) cells and T lymphocytes (helper and cytotoxic) secrete interferon IFNγ, leading to a reduction of angiogenesis and proliferation of cancerous cells; moreover, macrophages and dendritic cells (DC) secrete cytokines that activate immune cells to phagocytize dead tumor cells.

Equilibrium phase: Residual cancerous cells remain in a dormancy state because DC and cytotoxic T cells secrete IFNγ and inhibitory cytokines (IL12), suppressing them.

Escape phase: Tumor cells change their features which will be transmitted to the daughter cells, therefore escaping immunosuppression and proliferating, along with the apoptosis of the effector immunocytes[36]. This process is illustrated in Figure 1.

The tumor mechanisms to evade suppression by the immune system may include a reduced expression of the tumor antigens and major histocompatibility class I (MHC) antigen, Fas-L modulation, increased synthesis of inhibitory cytokines such as TGFβ1, IL10, IL6, VEGF, prostaglandin, and an increased number of T regulatory lymphocytes (Treg) and myeloid-derived suppressor cells (MDSC)[37].

Tumor immunosuppressive microenvironment
The most important components of the tumor immunosuppressive microenvironment are represented by the regulatory T cells (Tregs) and mesenchymal- or bone marrow-derived stem cells (BM-MSCs). Tregs represent CD4+CD25+FOXP3+ T lymphocytes that determine reduced activity of cytotoxic and helper T cells, and of NK cells and are involved in the immunological tolerance to self-antigen and the persistence of *Helicobacter pylori* (*H. pylori*)-related inflammation[38]. BM-MSCs migrate to cancerous tissues and, in some animal models, were shown to create an immunosuppressive environment in chronic *H. pylori* infection and to represent a "seeding point" for gastric carcinogenesis[39-41]. In this regard, immunotherapeutic strategies directed against Tregs and BM-MSCs and against immunosuppressive cytokines seem to be promising.

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed death-1 (PD-1) play essential roles in the immune checkpoint modulation. Normally, these molecules modulate the response of T lymphocytes to antigens. CTLA-4 represents an inhibitory receptor exhibited by T cells, whereas PD-1 represents a co-inhibitory receptor located on the cell surface, suppressing T cell activity in peripheral tissues in the context of inflammation. PD-1 is widely expressed on T and B lymphocytes, monocytes, and natural killer cells; conversely to CTLA-4, PD-1 is involved in subsequent phases of immune responses[42]. It has been shown that various tumor cells upregulate PD-L1. In gastrointestinal cancers, PD-L1 upregulation has been identified in pancreatic, gastric, and colorectal cancers, correlating in several studies with a poor prognosis[43,44].

Significance and prognostic role of tumor-infiltrating lymphocytes in gastric cancer
Tumor-infiltrating lymphocytes (TILs) comprise the presence of T cells, B cells and NK cells[45]. T cells include cytotoxic lymphocytes (CD8+), helper T cells (CD4+), memory T cells (CD45RO+) and T regulatory cells (FOXP3+). Specific cell membrane antigens of TILs bind to specific cellular types: CD3, CD4, CD8 and FOXP3 bind to T cells, CD20 to B cells and CD57 to NK cells[46].

In the complex relationship between the host immune microenvironment and cancer occurrence and progression, TILs seem to gain bidirectional regulation abilities. In one way, tumor neoantigens captured by the DC are presented on MHC molecules to the T cells, leading to the activation of effector T cells, with subsequent infiltration of the tumor and destruction of the cancerous cells. In addition, these activated cells secrete inhibitory cytokines, with the augmentation of antitumor effects[47,48]. On the other hand, TILs may help cancer to proliferate, either by creating an appropriate environment for tumor growth or by protecting tumor cells to survive[49].

The stromal TILs represent the mononuclear inflammatory cells infiltrating tumor stroma, whereas intratumor TILs define the intraepithelial lymphocytes/mononuclear cells within the tumor. Stromal TILs were shown to predict disease-free survival (DFS) of patients[50].

The assessment of TILs as a prognostic biomarker in gastric cancer patients has led to controversial conclusions. The presence of various subsets of cells seems

Figure 1 The three phases of cancer immunoediting. A: Phase 1: Elimination; B: Phase 2: Equilibrium; C: Phase 3: Escape. NK: Natural killer cells; Th: T helper lymphocyte; Tc: T cytotoxic lymphocyte; T reg: Regulatory T lymphocyte; DC: Dendritic cell; CC: Cancer cell.

to differently influence the patient's prognosis. Studies have shown that high density of intratumor TILs are associated with better prognosis (HR = 0.55)[51-53].

Additionally, some data in the literature revealed that an increased number of CD8^{+} T cells, both intra- or extra-tumor located, is associated with an improved DFS and OS[54-57]; in contrast, the results of a recent study showed that an increased number of CD8^{+} cells correlate with poor overall survival and increased expression of programmed death ligand 1 (PD-L1)[58]. Other studies showed that a high density of intratumor FOXP3^{+} Treg is correlated with a poor OS, whereas an extratumor high density of this cell type leads to an increased OS[59-64]. The increased intratumor Treg/CD8^{+} lymphocytes ratio is correlated with a decreased OS[65] and the presence of T helper 17 and T helper 22 with tumor progression[66]. Moreover, an increased T helper 1/ T helper 2 of CD4^{+} T lymphocytes represents a favorable prognostic factor[67]. A better OS was associated with an increased intratumor presence of various immunocytes, such as CD3^{+} T cells[57,68], CD57 NK [51,57,69,70], CD45RO^{+} (memory T cells)[71], and T-bet^{+} (marker for T helper 1 lymphocytes)[72]; in addition, an increased DFS was observed in the case of high intratumor density of CD20 (surface marker of B cells)[73]. The data show a decrease in CD3^{+} TILs density along with tumor progression[74]. On the other hand, the subgroup of CD45RO^{+} T lymphocytes seems to prevent peritoneal spreading of gastric neoplasias[75]. All the data from the literature demonstrate that high densities of CD8^{+}, CD3^{+}, and CD57^{+} TILs and low densities of FOXP3^{+} TILs represent favorable prognostic factors in gastric neoplasia.

CD4^{+} T cells secrete various cytokines, such as IL-17, the role for which the data from the literature reveal controversial results. Some of the studies showed that this cytokine could stimulate tumor angiogenesis, growth, and spreading[76], while other studies show that IL-17 exhibits anticancer effects, either by stimulating the cytotoxic activity of TIL[77] or by stimulating the maturation of DC[78].

The study of Yuan *et al*[79] revealed that gastric cancers present an increased percentage of CD4^{+} T lymphocytes and lower CD8^{+} T cells (with an increased CD4^{+}/CD8^{+} ratio) compared to blood and, further, to paraneoplastic tissue. In addition, the number of TILs of effector and memory T cell type is significantly higher than in the case of circulating T cells. As we have already mentioned, the involvement of Tregs in antitumor immunity is controversial, and their role may differ according to the type of cancer[80,81]. These authors considered CD4^{+}CD25 [high]CD127^{low} as being the most specific marker to define the Treg population, with the percentage of these cells being increased among TILs, demonstrating the accumulation of immunosuppressive Tregs at the site of a gastric tumor[79]. Recent studies show that the coexpression of PD-1^{+} and Tim-3^{+} define the most hypo-functional T lymphocytes[32,82]. The percentage of these cells among TILs was significantly increased in gastric tumors, especially in patients with advanced stages, suggesting that they may be implicated in a tumor immune escape phenomenon and that TILs in gastric cancer show T cell dysfunction. The combined blockade of these molecules seemed to have a

synergistic effect on IFNγ production and, therefore, may provide new promising immune modulating strategies[79].

Finally, the strategies of immunotherapy in gastric cancer are directed on TILs and are based on augmenting anticancer immunity by blocking the interaction of CD8+ T lymphocyte-related receptors such as CTLA-4 and PD-1 and their ligands situated on tumor cells (PD-L2 and PD-L1). Furthermore, it may include the stimulation of the local immune response by targeting and modulating different subsets of CD8+ TILs.

Significance of tumor infiltrating DCs in gastric cancer

Immunotherapy needs the activation of the cellular immune responses following the presentation of the tumor antigen peptides by DCs to T cells[83].

It has been demonstrated that the density of tumor infiltrating DCs correlates with the staging and prognosis in gastric neoplasias, and patients with a high amount of DCs present an improved OS compared with patients with a lower density of DCs[69]. The study of Tsujitani *et al*[84] showed that the use of postoperative adjuvant immunotherapy exhibits beneficial results on the survival of gastric cancer patients with reduced tumor DC infiltration.

Classification of the immune microenvironment of the gastric cancer according to the presence of CD8+ TILs and PD-L1 expression

Based on the existence of tumor-infiltrating lymphocytes (TILs) and PD-L1 expression, it has been proposed to categorize the tumors into four types[85] as follows: type I (TILs+ PD-L1+), presenting adaptive immune resistance; type II (TILs- PD-L1-), revealing immune ignorance; type III (TILs- PD-L1+), showing intrinsic induction; and type IV (TILs+ PD-L1-) in which other suppressors have a role in initiating immune tolerance. This stratification may have a certain prognostic role and may guide efforts towards a specific immunotherapeutic strategy.

Because tumor response to immunotherapy using PD-1 blockade requires the presence of CD8+ TILs that are downregulated by PD-1/PD-L1 activity[86], this type of therapeutic strategy in type I cancer might improve prognosis. As tumors included in type II and III lack TILs, the combination of immune checkpoint inhibitors with a vaccine that is capable to induce T cell activation, migration and infiltration at the tumor site might improve the clinical outcome in these patients[87].

Correlation between TILs and gastric cancer subtypes

Among the four molecular subtypes of gastric cancer, EBV+ and MSI tumors often show the activation of immune mechanisms, being associated with a high density of TILs, which has been correlated with an improved cancer-specific survival[88]. An increased number of CD8+ and FOXP3+ TILs were associated with improved OS in MSI-H gastric cancers[55] and EBV-associated cancers[87]. In addition, another study showed that a high number of CD3+ and CD8+ TILs in EBV-associated and MSI gastric cancer subtypes are associated with better survival[56].

Advanced TNM stages of EBV+ tumors were correlated with a reduced density of CD4+, CD8+ and Foxp3+ TILs and PD-1 expression. Additionally, PD-L1 expression was shown to predict a reduced survival in EBV-associated cancers. Approximately two-thirds of EBV+ gastric cancers were proved to present a type I or IV microenvironment associated with a better prognosis by inducing adaptive immune responses (type IV showed the best 5-year OS), whereas more than 70% of negative EBV tumors belong to the type II and III microenvironment, showing an absence of an immune response and a poor prognosis[87].

All these results are indicating the possibility of different subsets of TILs to be used as prognostic markers in these specific categories of patients. Moreover, EBV-associated and MSI gastric cancer categories might become potential targets of immunotherapy.

Significance of peripheral immune status

Myeloid-derived suppressor cells (MDSCs) represent immune suppressive cells with the ability to inhibit T cell (CD4+ and CD8+) activation and increase T cell apoptosis[89]. The data showed that high intratumor density of MDSCs is related to a poor prognosis in gastric cancer[80,90]. Peripheral blood granulocyte MDSCs are significantly increased in cancerous patients[91]. Shoji *et al*[92] noted that an increased proportion of granulocyte MDSCs prior to chemotherapy represents a negative prognostic factor for PFS in advanced gastric cancer patients receiving cisplatin-based chemotherapy and tends to be associated with poor OS.

Several papers showed that IL-8 is involved in gastrointestinal carcinogenesis by its ability to recruit MDSCs[93]. In addition, an increased number of circulating MDSCs was associated with advanced tumor stages, increased serum IL-8 levels, and dysfunction of T cells[94]. In this light, IL-8 seems to determine an adverse immune status[92].

Currently, numerous papers target defining the host inflammatory response to cancer. Due to a release of cytokines, the systemic inflammatory response seems to be responsible for the promotion of angiogenesis, DNA alteration, and tumor proliferation[95].

Studies have demonstrated that the neutrophil to lymphocyte ratio (NLR) has a prognostic value in patients with solid cancers[96-98]. In oncologic patients, lymphopenia is a marker of deficient cell-mediated immunity, while neutrophilia is a sign of response to systemic inflammation. Furthermore, malnutrition/ hypoalbuminemia show correlation both with immune suppression and systemic inflammation, which are phenomena overexpressed in advanced tumor stages[99,100]. Gonda *et al*[101] consider that NLR might be a useful biomarker for assessing tumor response to chemotherapy, immune suppression, malnutrition and unfavorable prognosis in gastric cancer patients. Moreover, it has been suggested that combining anti-inflammatory agents with chemotherapy provides an enhanced efficiency of the treatment.

HISTORY OF IMMUNOTHERAPY

The concept of immune modulating treatment was used for the first time by Edward Jenner (1798), who demonstrated that inoculating humans with cowpox could prevent smallpox occurrence. Over time, this strategy was implemented in developing serum and vaccinations. The efficacy of immunotherapy in cancer treatment was demonstrated by WB Coley (1891) who, by injecting streptococcal germs into a patient with unresectable cancer, determined the decrease of the tumor size[102]. Furthermore, Ehrlich (1909) has suggested that the host immune system could suppress the tumor growth by recognizing cancerous cells as non-self. Half a century later, Burnett has proposed the theory of tumor immune surveillance[35], recently completed by Schreiber *et al*[49] with the concept of cancer immunoediting.

In recent years, many immunotherapeutic strategies have been developed, including treatments using monoclonal antibodies, cytokines, cytotoxic cells, T cells infusions and gene transferred vaccines[36], having the aim of either increasing the host antitumoral response capacity or increasing the immunogenicity and susceptibility to treatment of the tumor cells[103].

CURRENT IMMUNOTHERAPEUTIC STRATEGIES IN GASTRIC CANCER

The development of an effective immunotherapeutic management for digestive cancers has evolved relatively slowly, with the majority of these immune-modulating approaches still under assessment in early phase clinical trials, mostly because of their well-known lack of antitumor effector T lymphocyte responses and their decreased immunogenicity[104]. Despite these specific aspects, some immunotherapeutic approaches, such as those using anti-PD/PD-L1 immune checkpoint inhibitors, proved to also be effective in cancers defined by a poor immunogenic nature[105].

Immunotherapeutic strategies may be classified into[42]:

Active immunization strategies, including: (1) Adoption of cytokines (*e.g.*, IFNγ, IL-10, IL-2) to date, leading to inconclusive results[103]. (2) Vaccination strategies that include: vaccines using peptides/proteins recognized by CD8+ and CD4+ lymphocytes, such as various tumor-rejection antigens (including melanoma-associated antigen (MAGE-3) or HER-2/neu)[106]; new vaccines using a compound formed by an immunogenic protein fused with peptide (Z12) that determines a persistent antitumor T cell response[107]; DC-based vaccines; RNA-based vaccines, *etc.* (3) Immune checkpoint inhibitors (anti-CTLA4, anti-PD/PD-L1). (4) Combination of different immunotherapeutic strategies. (5) Combination of immunotherapy with standard treatment.

Passive immunization strategies: Adoptive cell therapy (ACT) using TILs- refers to the passive transfer of antitumor T lymphocytes into a tumor-bearing host followed subsequently by the direct destruction of this cancer[108]. Current immunotherapeutic strategies are presented in Figure 2.

Cellular immunotherapies in gastric cancer

Currently, immunotherapy in gastric cancer patients includes cell-based strategies aimed either to activate cytotoxic T lymphocytes directed against cancer cells or to bind molecules expressed by tumor cells.

ACT: This technique refers to injection of different tumor-specific T lymphocytes into a cancer patient, such as cytokine- and anti-CD3 monoclonal antibody- induced killer cells, as well as TILs.

(1) Cytotoxic T cell therapy

In a preclinical study, cytotoxic activity of peripheral blood lymphocytes extracted from gastric tumor patients or from healthy individuals was induced using different HLA-A matched allogeneic gastric cancer cells, exhibiting antitumor efficacy against HLA-A2 and HLA-A24 gastric cancer cell lines[109]. Furthermore, Kawamoto *et al*[110] demonstrated the efficiency of peptide-based immunotherapeutic strategies by proving that cytotoxic T lymphocytes were able to kill HLA-A-0201/2402 colon and gastric cancer cells, which were positive for mitotic centromere-associated kinesin (MCAK) (a new cancer antigen) in an HLA-Ⅰ restricted way. In addition, MHC-1 restricted T cells were obtained from primary tumors, metastatic lymph nodes, and ascites of autologous gastric cancer and were proved to detain different recognition characteristics towards gastric cancer antigens[111]. Moreover, splenic MAGE-specific cytotoxic T lymphocytes against HLA-A2 cancer cell antigen, existing in testis and several neoplasias (including gastric cancer), were successfully obtained and tested[112].

Another preclinical study has revealed that cytokine-induced killer (CIK) cells, mostly by stimulating IFN-γ and tumor necrosis factor-alpha, exhibit antiproliferative effects against the MGC-803 gastric cancer cell line[113]. In addition, Kim *et al*[114] demonstrated the benefits of using ACT with CIK cells in gastric cancer patients. These CIK cells, isolated from the human peripheral blood mononuclear cells and activated by IL-2 and anti-CD3 antibody, were able to suppress the MKN74 human gastric cancer cell line *in vitro* and inhibit tumor proliferation in a nude mouse model.

As gastric cancers usually show paucity of stroma infiltration, *in vivo* studies have suggested administration of immunotherapy using ACT combined with chemotherapy[115]. Furthermore, the chemotherapeutic drug oxaliplatin, by stimulating high-mobility group box 1 protein to induce anticancerous T lymphocytes, is capable of producing an immunogenic cancer cell death[116]. In several *in vitro* and *in vivo* studies on drug-resistant gastric cancer, a high amount of cytokines was

Figure 2 Current immunotherapeutic strategies in gastric cancer. NK: Natural killer cells; TILs: Tumor-infiltrating lymphocytes; CIK: Cytokine-induced killer cells; ACT: Adoptive cell therapy; DCs: Dendritic cells; PD-1: Programmed death-1; PD-L1: Programmed death ligand-1; CTLA-4: Cytotoxic T-lymphocyte-associated antigen 4; CI: Checkpoint inhibitors.

induced by combining this drug with CIK cells[117]. It was suggested that chemotherapy associated with T lymphocyte reduction would be capable of enhancing the results of ACT therapy by stimulating the persistence of endogenous T cells in circulation, while depleting autoimmune reactions on healthy tissues. However, these results unfortunately occurred at the expense of severe infectious adverse events in these patients[118].

Moreover, the results obtained by combining ACT treatment with an antibody directed against both anti-epidermal growth factor receptor (EGFR) and anti-epithelial cell adhesion molecule (EpCAM), that specifically targets the simian virus 40 (SV40) T antigen-specific T cells (previously transduced with a truncated human EGFR), showed a better tumor reduction and OS than with ACT alone [119].

Du *et al*[120] used a mouse model of gastric cancer and concluded that ACT by peritoneal injection of CIK might be both a beneficial and minimally invasive strategy for treating this type of cancer.

The first clinical trial proving the beneficial results of ACT in humans used lymphokine-activated killer cells plus IL-2 in patients with metastatic melanoma, leading to the approval of the treatment for this group of patients[121-123]. Furthermore, this strategy determined a significant tumor reduction in patients with different tumors[124].

Zhang *et al*[125] showed that administration of expanded activated autologous lymphocytes, which were stimulated by anti-CD3 monoclonal antibody (mAb) and IL-2, to gastric neoplasia patients led to a prolonged OS compared to the group that received only standard chemotherapy.

The efficiency assessment of combined treatment using CIK cells and chemotherapy as adjuvant therapy in stage Ⅱ/Ⅲ of gastric neoplasia after curative gastric resection revealed a significantly prolonged OS *vs* the group using chemotherapy alone[126]. In a similar context, a clinical trial analyzed the possible toxicities of adjuvant ACT associated with chemotherapy (including 5-fluorouracil/capecitabine), showing an improvement in both DFS and OS, without the development of severe adverse reactions[127]. Moreover, a clinical trial assessing this type of combined adjuvant treatment in stage Ⅲ/Ⅳ (M0) gastric cancer patients after R0/D2 resection showed a significantly longer 5-year OS and DFS *vs* adjuvant chemotherapy[128].

In cases of advanced gastric cancer, several clinical studies have also proven an increased response rate, better quality of life and even an increased OS in patients treated with chemotherapy (FOLFOX4) plus CIK cells *vs* chemotherapy alone[129,130].

By assessing the administration of a chemotherapeutic regimen followed by autologous CIK cells, the results highlighted an improved remission rate in gastric cancer patients, associated with tolerable and reversible side effects. Standard chemotherapy using XELOX plus CIK cells administered intraperitoneally in gastric cancer patients showed a marked decrease in ascites volume and OS prolongation[131].

In a pilot study, patients with advanced gastric cancer, who received gamma delta T cells with zoledronate intraperitoneally as a local treatment for carcinomatous ascites, showed a significant reduction both in the number of peritoneal tumor cells and ascetic volume with no serious side effects[132].

Several clinical trials are currently investigating the tumor responses after adjuvant administration of ACT plus chemotherapy after surgical resection in advanced gastric cancer patients[133]. In a phase Ⅱ clinical study involving gastric cancer patients in stages Ⅰ-Ⅲ, the adjuvant combination of autologous tumor lysate-pulsed dendritic and CIK cells (Ag-D-CIK) plus chemotherapy is currently being evaluated following curative resection[134].

A clinical trial is currently investigating the safety and efficiency of infusing chimeric antigen receptor (CAR) T cells specific for EpCAM into relapsed or refractory gastric cancer patients[135].

A phase Ⅰ/Ⅱ clinical trial is currently investigating the benefits of infusing CAR T cells targeting mucin 1 (MUC1) in several solid cancers (including gastric tumors), as its overexpression leads to chemotherapeutic-refractory tumors[136].

A phase Ⅰb clinical trial on advanced gastric cancer expressing CEA assesses the efficacy of injecting anti-CEA CAR T cells into the hepatic artery targeting liver metastasis[137].

Additionally, a phase Ⅰ/Ⅱ clinical study on HER2^{+} gastric cancer patients (defined as HER2 in immuno-histochemical tumor tissue greater than or equal to 2 levels) with liver metastasis is analyzing the cytotoxic potency of engineered pluripotent stem cells and T cells, which specifically bind to HER2[138]. In addition, another phase Ⅰ clinical trial assesses the safety profile of administrating autologous T cells equipped with a bi-specific antibody (HER2 Bi-Armed T cells) in gastric and esophageal neoplasias[139].

Patients with metastatic gastric cancer are also investigated regarding a combination of S-1 plus dendritic cell activated CIK (DC-CIK) (phase Ⅰ/Ⅱ clinical trial)[140].

(2) Adoptive immunotherapy using TILs
The use of ACT with TILs is not associated with an immediate effect because this therapeutic protocol requires approximately six wks, as T cells must undergo the following preparation steps before infusion: first, they are isolated from tumor tissue; next, they are *in vitro* expanded; and finally, tumor-specific T cells are selected[141].

The immunotherapy using TILs has led to encouraging results in preclinical models[142], but not in all clinical studies (except for melanoma)[143,144].

In most gastric cancer patients, TILs exhibit a specific type-1 T cell response to cancer antigens. It is important to note that in order to obtain "*in vivo*" destruction of the tumor cells, the efficacy of tumor-specific T cells usually needs to be enhanced by combining vaccination using specific cancer antigens/peptides or by injecting *in vitro* expanded autologous cancer-specific T cells[103].

Moreover, TILs can sometimes stimulate proliferation of tumor cells. Studies show that HP0175-specific TILs in gastric cancer patients infected with *H. pylori* determine gastric Th17 response, exhibiting a pro-inflammatory low cytotoxic TIL response; Th17 cells promote tumor progression through the promotion of inflammation by secretion of IL-17 and other interleukins, which could induce proliferation and migration of cancer cells; therefore, TILs reveal a correlation between *H. pylori* infection and gastric cancer development[145].

Because studies have demonstrated that a high Tregs/CD8^{+} ratio in the tumor areas represents an independent factor for poor OS, a combination of the deletion of Tregs plus the stimulation of effector T cells may represent an effective immunotherapy in gastric cancer patients[65].

ACT using TILs isolated from the patient's tumor was also assessed in cases with gastric neoplasia[146]; the results of a clinical trial showed a longer OS using a combined treatment of ACT using TILs and standard chemotherapy *vs* chemotherapy alone[143].

(3) Adoptive immunotherapy using NK cells
The data show that NK cells exhibit cytotoxic activity against allogeneic and autologous cancer cell lines, including gastric cancer cells lines[147], and could prevent tumor metastatic spreading[148,149]; additionally, intra-tumor infiltration of NK cells is associated with a longer survival in neoplastic patients[150]. Patients with advanced gastric adenocarcinoma having a high density of NK cells demonstrated a prolonged survival rate *vs* those with the low density NK[151]. The number of apoptotic NK cells (Fas^{+} NK cells) is significantly higher in gastric neoplasia patients *vs* normal controls and is correlated to the tumor progression[152].

Clinical data revealed a favorable prognostic role of NK cells in gastric cancer patients, with a high level of CD57 antibody expression in gastric tumors correlated with a reduced size of tumors, N0 tumors, more surgical resections and prolonged 5-year OS[51].

By culturing autologous peripheral blood mononuclear cells with K562 cells, researchers were able to obtain cytotoxic NK cells from cancer patients[147], suggesting a possible role of immunotherapy using autologous expanded NK cells in clinical practice. In this regard, a clinical study using a combination of cell-based immunotherapy with autologous NK cells, γδ T cells, and CIK cells plus chemotherapy showed a statistically significant improvement of the 2-year progression-free survival and quality of life, but without demonstrating a significantly prolonged OS in gastric cancer patients[153].

The safety and efficacy of therapy with trastuzumab and NK cells in the treatment of gastric cancer is currently assessed in a clinical trial[154].

It has been demonstrated that lupeol, which exhibits a curative effect on various diseases, has the ability not only to stimulate the proliferation and the cytolytic activity of NK cells against gastric tumor cells, but also to inhibit the proliferation of some gastric cancer cell

lines. Therefore, this agent might be included (either alone or in combination with ACT) using NK cells in the therapeutic strategies of gastric tumors[155].

Cancer vaccines

Cancer vaccines have the aim of activating and increasing the number of effector T lymphocytes, leading to the augmentation of existing immunity, development of novel immunological response, and therefore an improved anticancer immunity[36].

Peptides derived from tumor-associated antigens [HER2/neu-derived peptide[156] and MAGE[157] are captured by antigen-presenting cells (such as DC)] and presented by means of MHC type Ⅰ for presentation to cytotoxic T cells, and by means of MHC type Ⅱ for presentation to T helper cells, determine their activation, followed by the destruction of tumor cells.

There are several types of vaccinations that have shown promising results in gastric cancer patients:

(1) HLA-A*2402-restricted URLC10-A24-177 and vascular epidermal growth factor receptor (VEGFR1-A12-9 1084) epitope peptide cancer vaccines in advanced chemotherapy-resistant gastric cancer patients - these are safe and induce enhanced specific cytotoxic T cell immune responses[158].

(2) Vaccination using survivin epitope peptide - a recent study suggested an excellent efficiency in gastric cancer patients upon inducing survivin-derived peptide-specific cytotoxic T lymphocytes from mononuclear cells isolated from blood of healthy individuals[159].

(3) Autologous gp96 vaccine in addition to chemotherapy as an adjuvant treatment in patients with resected gastric cancer (phase Ⅱ study) - glycoprotein (gp) 96, belonging to the group of autologous tumor-derived heat shock proteins (HSPs), binds tumor-associated antigens, constituting the HSPs-peptide complexes that promote the activation of APCs, as well as the release of various cytokines, enhancing T cell antitumor immune response; the vaccination seemed be well tolerated and is associated with a potential for prevention of gastric cancer recurrence[160].

(4) Trials using DC-based anticancer vaccines - *ex vivo* expanded DCs were able to generate antigen-specific T lymphocytes responses both in animal models[161] and in clinical trials[162]: (1) vaccination using dendritic cells pulsed with HER-2/neu peptide generated tumor shrinkage (phase Ⅰ study)[143]; (2) vaccination using DC pulsed with nanoparticles MAGE-3 peptide-loaded stopped tumors from proliferating in a mouse model of gastric cancer (phase Ⅰ study)[163]; and (3) vaccination with cancer-loaded autologous DCs (stimulated by autologous apoptotic gastric tumor cells) determined the activation of memory T cells[164].

RNA-based DC vaccines: By using stabilized mRNA, DCs transfected with mRNA coding for tumor-associated antigen/whole tumor RNA were able to generate potent immune responses both in mouse models[165,166] and

clinical studies involving melanomas and renal cell carcinomas[167,168].

These RNA-based vaccines seem to present some potential benefits over the classical vaccination techniques: they are pharmaceutically safer because of the presence of transient and cytosolic active mRNA (lack of genomic integration); they have the ability to target multiple tumor-associated antigens; they are not associated with severe adverse events; and they are not MHC-restricted[36].

Because the clinical efficiency of DC vaccines is limited because of the short survival of DCs, mostly due to the cytolytic properties of DC-activated CD8+ T cells[169], their efficiency has been increased by using small interfering RNA (siRNA)-targeting phosphatase and tensin homolog (PTEN), which is involved in negative feedback in the signal transduction of the PI3K/AKT pathway[170]. This technique has increased the number of cancer-specific cytotoxic T cells and the antitumor immune response[171].

The results were improved by targeting the immunosuppressive IL-10 receptor in association with the siRNA DC vaccine[172], by using GM-CSF gene-modified DC[173], and by removing Tregs along with DC vaccination[174].

Strategies of combining vaccination with chemotherapy: The combination of an adjuvant Bacille Calmette-Guérin (BCG) vaccine with chemotherapy *vs* chemotherapy alone resulted in a better OS in patients with radically resected locally advanced gastric cancer[175].

Vaccination using gastrin-17 diphtheria toxoid (G17DT)-targeting gastrin peptide combined with chemotherapy (cisplatin plus fluorouracil) increased the OS of patients with advanced gastric cancer (phase Ⅱ clinical trial)[176].

Vaccination using peptides derived from human VEGF receptors 1 and 2 combined with standard chemotherapy (S1+ cisplatin) improved the OS in patients with advanced gastric cancer[177].

Literature data show that vaccination is a safe and tolerable strategy that is associated with better prognosis in gastric cancer patients, especially when it is performed in addition to classical chemotherapy.

Immunotherapy using checkpoint inhibitors

Immune checkpoints represent inhibitory pathways that are critical for maintaining self-tolerance and physiological homeostasis by controlling the intensity of physiological immune responses to prevent tissue injury, particularly when the immune system is fighting an infection. Additionally, they may also allow immune escape of cancer cells[36].

Immune checkpoint molecules, such as cytotoxic T lymphocyte protein-4 (CTLA-4) and programmed cell death protein-1 (PD-1), are involved in the inhibition of T cell activation via different pathways.

CTLA-4 is a co-inhibitory molecule exhibited on activated T lymphocytes and T regulatory cells, whose receptor on T cells interacts with its B7-1/B7-2 ligands

located on antigen-presenting cells (APCs), subsequently suppressing the T cell stimulatory signal mediated by CD28[178]. CTLA-4 expression is stimulated only in the context of T cell activation; afterwards, it competes with CD28 to bind to B7 molecules and decrease the immune response. By inhibiting this interaction using an anti-CTLA-4 antibody, T cell activation and proliferation is promoted, along with a decrease in immunosuppressive Treg cells among TILs[179].

PD-1 represents a co-inhibitory receptor that is found on the surface of several types of cells, such as activated T cells, Treg cells, and monocytes. It has two ligands, PD-L1 and PD-L2. PD-L1 is expressed on both immune and tumor cells, while PD-L2 is mostly expressed on APCs. In tumors, PD-L1 that is expressed on tumor cells binds to PD-1 on activated T cells that reach the tumor and generates a suppression signal for the activation of T cells, which become unable to destroy tumor cells, leading to a decrease in both cellular and humoral immune responses[180,181]. Unlike CTLA-4, which is considered to be necessary for T cell activation, the PD-1/PD-L1/2 pathway seems to protect tumor cells from attack by T lymphocytes. It has been demonstrated that by inducing antibody-mediated blockage of the PD-1/PD-L pathway followed by the inhibition of this checkpoint, treatment is able to enhance the anticancer immune response of the host[22,182].

Several genetic studies have shown a possible correlation between PD-1 or CTLA-4 polymorphisms and the development of gastric cancer[183-185]. Additionally, the role of CTLA-4 gene promoter hypermethylation has been demonstrated as a risk factor for gastric tumor development, with CTLA-4 expression being significantly higher in gastric tumor samples *vs* normal tissue[186].

Prognostic significance of PD-1/PD-L1 and CTLA-4 expression in gastric cancer: Saito *et al*[187] showed that PD-1 expression in CD8^{+} and CD4^{+} T cells in gastric tumors was significantly higher *vs* in normal gastric mucosa. Wu *et al*[188] showed that PD-L1 expression was encountered in 42% of gastric cancer tissues but not in normal gastric mucosa.

A meta-analysis by Gu *et al*[189] that was based on 15 studies (most of them conducted in Asia) including 3291 patients, showed a high variability of PD-L1 immuno-histochemical expression among studies, ranging between 14.3% and 69.4%, due to the differences in cut-off values (between > 1% and > 50%).

Unlike in other tumors, such as lung cancer or melanoma, there is scattered PD-L1 expression in gastric cancer cells, which mostly occurs in infiltrating myeloid cells at the tumor invasive front[25,58].

There are several papers suggesting that PD-L1 expression in tumor cells may be upregulated by genomic alterations and oncogenic signaling, either through the phosphatidylinositol-3-kinase-protein kinase B (PI3K-AKT) or signal transducers and activators of the transcription (STAT) 3 pathway. Moreover, PD-L1 is upregulated by the microRNA-200/zinc-finger E-box-binding

homeobox 1 (ZEB-1) axis, which is closely related to epithelial-mesenchymal transition (EMT) conversion and to IFN-γ produced by TILs[190-192]. Mimura *et al*[193] showed that membranous PD-L1 immunohistochemical expression in gastric tumor cells was significantly correlated with the number of CD8^{+} TILs and IFN-γ positive cells in the tumor. Additionally, an enhanced cytotoxic effect of anti-PD-L1 monoclonal antibody treatment was observed after prior IFN-γ exposure.

Literature data regarding the expression of PD-1/PD-L1 as a prognostic in gastric cancer showed controversial results. Dai *et al*[53] reported an association between PD-L1 expression and an increased density of TILs; this increased density of TILs and higher levels of PD-L1 mRNA in gastric cancer was significantly correlated with a better prognosis. Some studies showed a significantly improved prognosis in patients with PD-L1 positive tumors[194,195]; conversely, others reported an association between PD-1/PD-L1 expression in cancer cells and TILs and advanced tumors, increased tumor size, the presence of deep invasion, lymph node metastasis, and perineural invasion and a significantly worse prognosis in these patients[196-198]; and the third group of papers found no influence of PD-L1 on the prognosis of gastric cancer[199].

A few recent meta-analyses have shown a correlation between PD-L1 and gastric cancer prognosis, demonstrating that PD-L1 overexpression is a worse prognostic factor in these patients[200-202]. Additionally, Zhang *et al*[203] performed a recent meta-analysis that included ten studies with 1901 gastric cancer patients and showed that PD-L1 expression was associated with a shorter OS and a poor clinicopathological status.

Fang *et al*[204] highlighted that PD-L1 was expressed both in tumor cells and in TILs. PD-L1 positivity in tumor cells was associated with differentiation, while its expression in TILs was correlated with a late stage of the disease, no surgery and the OS. Patients with PD-L1[+] TILs had a significantly poorer 5-year OS than those without PD-L1 expression (14.2 *vs* 18.3; *P* = 0.001). A study by Gao *et al*[205] showed that PD-L1 and PD-L2 positivity in primary tumors and metastatic lymph nodes decreased the number of CD8^{+} T cells, and the amount of PD-1 positive expression on CD8^{+} T cells in primary tumors were prognostic factors that were correlated with a poor prognosis in stage Ⅱ/Ⅲ gastric cancer patients.

Again, the results of the meta-analysis by Gu and collaborators[189], showed that gastric cancer patients with deeper tumor infiltration, lymph node metastasis, venous invasion, and EBV[+] and MSI subtypes were more likely to be PD-L1 positive. Moreover, for the subgroups of Asian patients and patients with stage Ⅱ/Ⅲ gastric cancer, cut-off values greater than 50% and cytoplasm/nuclear PD-L1 expression within tumor cells were positively associated with the OS. The results of this meta-analysis demonstrated that PD-L1 overexpression represented a significantly adverse prognostic factor in gastric cancer, fitting the theory of the cancer immunity cycle[47].

Many other studies[32,206] have also demonstrated

that EBV^{+} and MSI gastric tumors tend to be positive for PD-L1 expression. These subtypes have a rich infiltration of lymphocytes, especially CD8^{+} T cells, in the tumor stroma; therefore, they may be categorized as medullary carcinomas. Moreover, in this context, PD-L1^{+} expression is associated with a significant increase in the number of CD8^{+} T cells at the tumor invasive front and with the ability of immune cells to infiltrate the center of the tumor[32]. Both EBV^{+} gastric cancers and MSI[+] gastric cancers have IFN-γ response genes, therefore, PD-1 pathway signaling seems to be a crucial mechanism for controlling a previous cytotoxic anticancer immune response[189]. The EBV[+] subtype is associated with amplification of the 9p24.1 locus, which harbors the PD-L1/PD-L2 genes; PD-L1 positivity is found in 50% of the cancer cells and 94% of immune cells in this subgroup[194,207]. Additionally, approximately 33% and 45% PD-L1[+] expression levels are encountered on tumors cells and immune cells, respectively, in MSI gastric tumors[208]. These data suggest that EBV[+] and MSI gastric cancers may be preferred candidates for PD-1 blockade immunotherapy. These types of neoplasias that are associated with rich inflammatory infiltrates are considered "hot" or inflamed tumors, while poorly immunogenic cancers are termed "cold." "Hot" tumors are characterized by the expression of immune-inhibitory signals, such as PD-L1, indoleamine-2,3-dioxygenase (IDO), and Treg cells[209,210], which counterbalance the effects of cytotoxic T lymphocytes. Therefore, using combined treatments to convert cold into hot tumors may increase the proportion of patients who benefit from immunotherapy[211].

While some data from non-small cell lung cancer (NSCLC) and other cancers have revealed that PD-L1 immunohistochemical positivity in cancer and/or in immune cells (bioptic specimens) is correlated with beneficial results after checkpoint inhibitor immunotherapy using monoclonal antibodies[212], other studies have shown tumor responses to PD-L1 therapies in tumors with PD-L1- cancer cells[213].

The location of PD-L1 expression in TILs situated at the invasive front or even at the tumor center may negatively influence its use as a biomarker. Furthermore, stromal rather than membranous expression of PD-L1 may be the cause for the slightly poorer responses to single-agent PD-1 checkpoint inhibitors in gastric cancer *vs* other tumor types[208].

Because there are variations in the techniques, the antibody clones used, and the cutoff values for PD-L1 positivity that limit cross-trial comparisons of different tumor types, including gastric cancer, some researchers have proposed harmonization of PD-L1 testing to standardize the results. Recently, Jiang *et al*[214] elaborated an immunohistochemical-based immunoscore on a cohort of 879 Chinese gastric cancer patients and demonstrated that a high immunoscore corresponded to lower recurrence rates and improved OS after adjuvant therapy.

Data suggest that there is racial and geographical variability in the tumor-immune microenvironment that is related to different responses to immunotherapy; for example, non-Asian gastric cancer patients have tumors that are rich in TILs and are associated with high CTLA-4 signaling[215].

The study by Schlößer *et al*[198] found positive CTLA-4 expression in the tumor microenvironment of 86% of gastric cancer patients that was correlated with poor OS.

Clinical trials using checkpoint inhibitors: Clinical trials using CTLA-4 and PD/PD-L1 checkpoint inhibitors are listed in Table 1.

(1) CTLA-4 checkpoint inhibitors
Tremelimumab
Tremelimumab (formerly ticilimumab, CP-675,206) is a fully human monoclonal antibody against CTLA-4 that received FDA approval for the treatment of mesothelioma. It was the first immune checkpoint inhibitor that was investigated in patients with gastroesophageal tumors.

A phase II study[216] investigated tremelimumab (at a dose of 15 mg/kg every 90 d, which is presently considered sub-therapeutic) in 18 patients with advanced esophageal, gastroesophageal junction or gastric adenocarcinoma. Although the median time to progression and OS were relatively short, at 2.83 and 4.83 mo, respectively, approximately one-third of patients were alive at one year. Currently, there is an ongoing clinical trial assessing whether tremelimumab is associated with the anti-PD-L1 antibody durvalumab; the dose of tremelimumab used as a monotherapy is 10 mg/kg every 4 wk[9].

Ipilimumab
Ipilimumab (trade name Yervoy, previously known as MDX-010 and MDX-101) was approved by FDA for the treatment of patients with unresectable/metastatic melanoma, after at least one line of systemic treatment has been performed (2011)[217]; the indications for this drug were recently extended to include pediatric patients (12 years and older) (2017) and adjuvant treatment of patients with cutaneous melanoma with pathologic involvement of regional lymph nodes greater than 1 mm in size who have undergone complete resection (including total lymphadenectomy); in addition, it was recently approved as the first-line treatment, in combination with nivolumab, for patients with intermediate or poor risk with advanced renal cell carcinoma (2018).

A randomized phase II trial enrolled patients with unresectable, locally advanced/metastatic gastric or gastro-esophageal junction cancer with partial response/stable disease after first-line chemotherapy with a combined fluoropyrimidine plus platinum regimen to receive either the best supportive care (consisting of continuation of fluoropyrimidine) or ipilimumab. The primary end-point of the study was immune-related PFS. Unfortunately, the study was ended earlier due to the lack of clinical efficiency of ipilimumab. The PFS was

Table 1 Clinical trials using CTLA-4 and PD/PD-L1 checkpoint inhibitors

Checkpoint inhibitors	Study number	Phase	Status	Study design	Study population	Primary outcome measures	Secondary outcome measures	No. of patients (estimated enrollment)	Final results
Nivolumab, Ipilimumab	NCT03342417	II	Recruiting	NIVO + IPI	-Neoadjuvant breast cancer -Platinum-resistant advanced ovarian/GC	- % AE	- DOR, OS, QOL, recurrence rate	60	Pending
Nivolumab, Ipilimumab	NCT02872116 (CHECKMATE-649)	III	Recruiting	- NIVO + IPI/ -NIVO + chemo *vs* chemotherapy (XELOX/FOLFOX)	-Naive advanced/metastatic GC/GEJ	-OS NIVO + IPI *vs* chemo (PD-L1 + tumors) -OS NIVO + chemo *vs* chemo -ORR, PFS nivo + chemo *vs* chemo	-OS NIVO + IPI *vs* chemo -PFS NIVO + IPI/ NIVO + chemo *vs* chemo (PD-L1+) -ORR NIVO + chemo *vs* chemo	1349	Pending
Nivolumab, Ipilimumab	NCT02935634 (FRACTION-GC)	II	Recruiting	- NIVO + IPI -NIVO + RELATLIMAB -NIVO + BMS-986205	-Advanced GC	-ORR, DOR, PFS	-% AE, SAE, discontinuation/death due to treatment	300	Pending
Nivolumab, Ipilimumab	NCT03044613	I b	Recruiting	- NIVO/NIVO + IPI prior to chemoradiation + NIVO	-Neoadjuvant treatment, resectable stage II/III EC/GEJ	- % AE	-Feasibility of induction treatment -Path CR -Quantity of NIVO bound to PD1 receptor -Changes in expression of immune markers -OS, RFS	32	Pending
Nivolumab, Ipilimumab	NCT03443856 (VESTIGE)	II	Not yet recruiting	- NIVO + IPI	-Adjuvant treatment GC/GEJ adenocarcinoma stage I b-IVa, ↑risk of recurrence (ypN1-3 + /R1) after neoadjuvant treatment + resection	-DFS	-OS -Relapse rate -Loco-regional/distant failure rates -% AE, QOL, global health status	240	N/A
Nivolumab, Ipilimumab	NCT03409848 (INTEGA)	II	Recruiting	-IPI/FOLFOX + NIVO and TRAS	-Advanced/metastatic GC adenocarcinoma, previously untreated	-OS	-% AE -PFS, RR, QOL -Translational research -Central imaging review	97	Pending
Nivolumab, Ipilimumab	NCT02834013	II	Recruiting	-NIVO + IPI	-Rare tumors, including gastric NET, SqCC, GIST	-ORR-RECIST	-Toxicities -OS, PFS -Clinical benefit rate -ir-ORR/PFS	707	Pending
Nivolumab, Ipilimumab	NCT03126110	I/II	Recruiting	-NIVO + INCAGN01876 -IPI + INCAGN01876 -NIVO + IPI + INCAGN01876	-Advanced/metastatic malignancies, including GC	-Toxicities (%AE) -ORR-RECIST	-DR, DDC, PFS, OS- RECIST	450	Pending
Nivolumab, Ipilimumab	NCT03241173	I/II	recruiting	-NIVO + INCAGN0949 -IPI + INCAGN0949 -NIVO + IPI + INCAGN0949	-Advanced/metastatic malignancies, including GC	-Toxicities (%AE) -ORR-RECIST	-DR, DDC, PFS, OS- RECIST	651	Pending

Drug	NCT number	Phase	Status	Treatment	Patient population	Primary endpoint	Secondary endpoint	N	Results
Ipilimumab	NCT01585987	II	Completed	-IPI *vs* BSC (5-FU)	-Unresectable/ metastatic GC/GEJ adenocarcinoma (following 1st line treatment)	-ir-PFS (ir RECIST) -% BOR	-PFS (mWHO) -OS	143	-ir-PFS ↓ (2.92 *vs* 4.89 mo); -mWHO-PFS↓ (2.72 *vs* 4.89) (P = 0.03); -OS at study completion 12.68 *vs* 12.06 mo
Tremelimumab, Durvalumab	NCT02658214	I	Recruiting	-TREME + DURVA + chemo (platinum-based SOC)	-1st line locally advanced/ metastatic solid tumor, including GC/GEJ	-Laboratory findings -%AE, safety, tolerability -Tumor assessment (RECIST)	-	42	Pending
Nivolumab	NCT03453164 (CIRCUIT)	I / II	Recruiting	NIVO + radiotherapy	-Unresectable recurrent GC (3rd line)	-DCR: -PD: on CT/ MRI/ PET-CT -CR/PR/SD	-Mean survival -% AE -Local control rate -% PL-L1+, MHCI- tumor cells -Cytokines serum concentration -% regT cells -% Ag-specific CTL	40	Pending
Nivolumab	NCT02267343	III	Active, not recruiting	NIVO *vs* placebo	-Refractory, unresectable, advanced/ recurrent GC/GEJ	-OS, PFS	-ORR, DOR, %AE, %SAE, safety	480	Pending
Nivolumab	NCT02746796	II / III	Recruiting	NIVO + chemo	-1st line therapy, unresectable advanced/ recurrent GC/GEJ	-PFS -OS	-ORR, DOR, DCR -TTR, BOR	680	Pending
Nivolumab	NCT03006705	III	Recruiting	NIVO + chemo *vs* placebo + chemo	-Adjuvant treatment *p* stage III GC/GEJ (after D2 resection)	-RFS	-% AE, SAE, laboratory abnormalities -OS -Safety- % AE, SAE, laboratory abnormalities	700	Pending
Nivolumab	NCT02999295	I / II	Recruiting	-NIVO + RAMUCIRUMAB	-Advanced/ recurrent unresectable GC/GEJ	-No. of pts with DLT -6 mo PFS	-%AE -ORR -DCR -OS, PFS	44	Pending
Nivolumab	NCT02946671	I	Recruiting	-NIVO + MOGAMULIMUMAB (KW-0761 = anti-CCR4)	-Preoperator treatment against solid cancers, including GC	-% AE -FOXp3 + tumors by immunohistochemistry	-ORR-RECIST -% ↓Treg	18	Pending
Nivolumab	NCT02951091 (Biomarker – integrated Umbrella)	Observational	Recruiting	-NIVO/ AFATINIB/ GSK2636771 + PACLITAXEL	-Advanced GC -Different molecular cohorts: PD-L1+, MSI-H, EBV⁺ → NIVO	-PFS	-	400	Pending
Nivolumab	NCT02465060 (The MATCH screening trial)	II	Recruiting	-NIVO/ other agents (according to genetic testing)	-Advanced/ metastatic solid tumors (including GC), lymphomas, multiple myelomas → mismatch repair deficiency (loss of MLH1/ MLH2)	-ORR	-OS, PFS -TTP	6452	Pending

Drug	NCT	Phase	Status	Treatment	Setting	Primary outcome	Secondary outcomes	N	Results
Nivolumab	NCT02862535	I b	Active, non-recruiting	-ANDECALIXIMAB (GS-5745) ± NIVO/chemo	-Previously treated, advanced GC/GEJ adenocarcinoma (Japan)	-Safety (% AE)	-Serum concentration of Andecaliximab -% Ab-anti Andecaliximab	36	N/A
Pembrolizumab	NCT03382600 (MK-3475-659/KEYNOTE 659)	II	Recruiting	-PEMBRO + OXALIPLATIN + TS-1 *vs* TS-1 -PEMBRO + CISPLATIN + TS-1	-Advanced CG/GEJ adenocarcinoma, HER2(-), PD-L1+	-ORR-RECIST	-ORR (i-RECIST) -DCR, DOR, TTR, PFS (RECIST, iRECIST) -OS, % AE	90	Pending
Pembrolizumab	NCT02901301	I / II	Recruiting	-PEMBRO + TRASTUZUMAB + chemo	-1 st line advanced, GC HER2+	-Recommended dose -ORR-RECIST	-DOR -TTR	49	Pending
Pembrolizumab	NCT03342937	II	Recruiting	PEMBRO + XELOX	-1 st line metastatic GC adenocarcinoma	-PFS	-OS -% AE	50	Pending
Pembrolizumab	NCT02918161	II	Recruiting	PEMBRO + chemo (SOC)	-Perioperative setting GC/GEJ	-2 years DFS	-Pathol CR -OS, ORR (RECIST) -DFS	40	Pending
Pembrolizumab	NCT02689284	I / II	Recruiting	PEMBRO + MARGETUXIMAB	-HER2 + advanced, metastatic GC/GEJ	-Expansion phase dose of Margetuximab -Antitumor activity: RD, ORR (RECIST, ir-RECIST)	-OS -PFS	72	Pending
Pembrolizumab	NCT03257163	II	Recruiting	-PEMBRO + CAPECITABINE + radiotherapy (perioperative)	-Mismatch repair deficient, EBV[+], operable GC	-RFS	-	40	Pending
Pembrolizumab	NCT03064490 (PROCEED)	II	Recruiting	-PEMBRO + chemoradiotherapy	-Neoadjuvant treatment, locally advanced EG cancers	-Pathol CR	-Toxicity (% AE)	38	Pending
Pembrolizumab	NCT02563548	1 b	Recruiting	-PEMBRO + PEGPH2O (Pegylated Recombinant Human Hyaluronidase)	-Hyaluronan-high (HA-H) patients with relapsed/refractory cancers (adenocarcinoma)	-ORR	-DCR, DOR, PFS (RECIST, ir-RECIST)	81	Pending
Pembrolizumab	NCT02954536	II	Recruiting	-PEMBRO+TRASTUZUMAB+chemo	-Advanced, metastatic HER2+, EG (1st line)	-PFS (RECIST)	-	37	Pending
Pembrolizumab	NCT03221426 (MK-3475-585) (KEYNOTE-585)	III	Recruiting	-PEMBRO + cemo *vs* placebo + chemo	-Neoadjuvant/adjuvant previously untreated GC/GEJ adenocarcinoma	-OS - EFS event-free survival) -Pathol CR -% AE + discontinuation of treatment	-DFS	860	Pending
Pembrolizumab	NCT03196232	II	Recruiting	-PEMBRO + EPACADOSTAT	-Metastatic/ unresectable GEJ	-6-mo PFS	-ORR (RECIST) -OS -RR	30	Pending
Pembrolizumab	NCT03019588 (MK-3475-063/KEYNOTE-063)	III	Recruiting	-PEMBRO *vs* chemo (PACLITAXEL)	-Progression after 1 st line platinum-fluoropyridine chemo, advanced GC/GEJ adenocarcinoma, PD-L1+ (Asia)	-OS, PFS (RECIST)	-ORR-RECIST -% AE, % discontinuation due to AE	360	Pending
Pembrolizumab	NCT03488667	II	Not yet recruiting	PEMBRO + mFOLFOX	-Neoadjuvant treatment GEJ adenocarcinoma -adjuvant treatment GC	-yp RR (pathologic response) -toxicity (% AE)	-ORR, DFS, OS -PET scan response rate -% PD-L1 + in tumor cells	40	N/A
Pembrolizumab	NCT03413397	II	Recruiting	PEMBRO + LENVATINIB MESYLATE	-Metastatic/ recurrent GC/GEJ	-ORR-RECIST	-PFS, OS -Characteristic immunologic changes	29	Pending

Drug	NCT	Phase	Status	Treatment	Population	Primary endpoints	Secondary endpoints	N	Results
Pembrolizumab	NCT02730546	I/II	Recruiting	PEMBRO + chemoradiotherapy	-Locally advanced, operable, GEJ/gastric cardia adenocarcinoma (neoadjuvant setting)	-Path CR -PFS	-R0 resection, DSF -Dose-limiting AE, % surgical complications, OS, PFS, time to relapse	68	Pending
Pembrolizumab	NCT02318901	I b/II	Active, not recruiting	PEMBRO-TRASTUZUMAB/ ADO-TRASTUZUMAB-ETAMSINE/CETUXIMAB	-Unresectable HER2+, advanced GC/GEJ	-Dose of mAb combined with PEMBRO	-% grade 3-4 AE -RR (RECIST, ir-RECIST) -OS, PFS -Circulating tumor DNA -Imaging changes	90	N/A
Pembrolizumab	NCT03095781	I	Recruiting	PEMBRO + XL888 (= Hsp90 inhibitor)	-Advanced gastrointestinal cancer (including GC)	-Recommended dose for combined treatment	-ORR, PFS, RS (RECIST) -OS	50	Pending
Pembrolizumab	NCT02346955	I	Terminated	-CM-24[MK-6018 = mAb against CEACAM1] ± PEMBRO	-Advanced/recurrent malignancies (including GC)	-% AE, discontinuation due to AE, DLT	-Maximum drug concentration, half-life elimination, ORR, DOR	27	Pending
Pembrolizumab	NCT02178722 (KEYNOTE-037/ ECHO-202)	I/II	Recruiting	-PEMBRO + EPACADOSTAT	-Selected carcinomas (including GC)	-% DTL -ORR	-PFS -% AE -OS	508	Pending
Pembrolizumab	NCT02903914	I/II	Recruiting	-PEMBRO + ARGINASE INHIBITOR INCB001158	-Advanced/ metastatic solid tumors (including GC)	-% AE	-Rrecommended dose of arginase Inhibitor ± PEMBRO -Pharmacokinetic profile -Antitumor activity of drugs (RECIST, ir RECIST)	346	Pending
Pembrolizumab	NCT03122548	II	Active, not recruiting	PEMBRO + CRS-207	-Recurrent/metastatic GC/EG (1-2 prior lines of systemic treatment)	-% AE	-Tumor response (RECIST) -OS -Characterization of immune response -Analysis of biomarker expression	79	N/A
Pembrolizumab	NCT02393248	I/II	Recruiting	-INCB054828 + PEMBRO/ chemo/ TRASTUZUMAB	-Advanced malignancies (including GC), progression after prior treatment	-Maximum tolerated dose, pharmacodynamic of INCB054828	-ORR -Maximum/minimum plasma Concentration of NCB054828	280	Pending
Pembrolizumab	NCT02494583	III	Active, not recruiting	-PEMBRO vs -PEMBRO + chemo vs -Placebo + chemo	-1 st line treatment, advanced GC/GEJ	-PFS (RECIST) -OS	-ORR, DOR (RECIST) -QOL	764	N/A
Pembrolizumab	NCT02370498 (KEYNOTE-061)	III	Active, not recruiting	-PEMBRO vs chemo (PACLITAXEL)	-Advanced GC/GEJ adenocarcinoma, Progressed after 1 st line (platinum + fluoropyrimidine), PD-L1+	-PFS, OS- in PD-L1+	-PFS, OS -TTP, ORR	592	Pending
Pembrolizumab	NCT02335411 (KEYNOTE-059)	II	Active, not recruiting	-PEMBRO or PEMBRO + chemo (CISPLATIN + 5-FU/ CAPECITABINE)	-Recurrent/ metastatic GC/GEJ adenocarcinoma	-% AE, discontinuation of treatment due to AE -ORR	-	316	Pending
Pembrolizumab	NCT03277352	I/II	Recruiting	-INCAGN01876 + PEMBRO + EPACADOSTAT	-Advanced/ metastatic malignancies	-% AE -ORR, CRR (RECIST)	-ORR, DCR, DOR, PFS, OS (rECIST, mRECIST)	166	Pending

Drug	NCT	Phase	Status	Treatment	Setting	Primary endpoint	Secondary endpoints	N	Result
Pembrolizumab	NCT02443324	I	Active, not recruiting	-PEMBRO + RAMUCIRUMAB	-GC/GEJ (NSCLC, transitional urothelial cancer, biliary tract cancer)	-DTL	-BOR of CR/PR, ORR -% SD -DOR, time to response -PFS, OS -Pharmacokinetics	155	Pending
Avelumab	NCT02625623 (JAVELIN GASTRIC 300)	III	Active, not recruiting	-AVE + BSC *vs* -chemo+BSC/BSC	-Unresectable, recurrent, locally advanced / metastatic GC/GEJ adenocarcinoma (3rd line)	-OS	-PFS, BOR -QOL	37	Pending
Avelumab	NCT03399071 (ICONIC)	II	Recruiting	-AVE + chemo (FLOT)	-Perioperative setting, operable EC/GC	-Pathol CR	-% grade 3-4 AE -Radiologic response (RECIST) -median PFS, OS	40	Pending
Avelumab	NCT02625610 (JAVELIN GASTRIC 100)	III	Active, not recruiting	-AVE maintenance *vs* 1 st line continuation of chemo (OXALIPLATIN + FLUOROPYRIMIDINE)	-Unresectable, locally advanced/ metastatic GC/ GEJ adenocarcinoma	-OS, PFS	-BOR (RECIST) -QOL -% AE	499	Pending
Avelumab	NCT01943461 (JAVELIN SOLID TUMOR JPN)	I	Active, not recruiting	-AVE	-Locally advanced/ metastatic solid tumors (Japan) → expansion part GC patients (Asia)	-DLT	-Concentration assessment, elimination half-life -% PD-L1 -BOR+ irBOR, PFS +irPFS -OS % Ab-anti AVE -% AE	57	Pending
Avelumab	NCT03475953 (REGOMUNE)	I / II	Not yet recruiting	-AVE + REGORAFENIB	-Advanced/ metastatic digestive solid tumors (including GC)	-Recommended doses -Assessment of Regorafenib antitumor activity -Pharmacokinetics	-Maximum tolerated dose -DLT -% AE -BOR, ORR, PFS, OS -Blood/tumor growth biomarkers	212	Pending
Avelumab	NCT02554812 (JAVELINE Medley)	I b/ II	Recruiting	-AVE + other immunotherapies → AVE + PD 0360324 (M-CSF mAb) (gastric cancer)	-Locally advanced/ metastatic solid tumors (including GC)	-% DLT -ORR	-Serum concentration of drugs -Ab-anti drugs -TTR, DOR, PFS, OS -Tumor biomarkers (PD-L1, CD8+T cells)	560	Pending
Durvalumab	NCT02734004 (MEDIOLA)	I / II	Active, not recruiting	-DURVA + OLAPARIB (PARP inhibitor)	-Advanced solid tumors (including GC)	-DCR (at CT/MRI) -% AE -Safety- vital signs, blood samples	-% PD-L1 -DCR (mRECIST) -Time to treatment discontinuation) -OS -% change in tumor size (CT/MRI) -Serum concentration of Ab-anti drug -Pharmacokinetics -ORR, DOR, PFS (mRECIST)	148	Pending

	NCT number	Phase	Status	Treatment	Population	Primary endpoint	Secondary endpoints	N	Results
Durvalumab	NCT02572687	I	Active, not recruiting	DURVA + RAMUCIRUMAB	-Locally advanced unresectable/ metastatic gastrointestinal (including GC/GEJ adenocarcinoma) and thoracic malignancies	-% DLT	-ORR, DCR -DOR, TTR -PFS -OS -Pharmacokinetics -% Ab-anti drug	114	Pending
Durvalumab	NCT02678182 (PLATFORM)	II	Recruiting	-DURVA *vs.* CAPECITABINE *vs.* TRASTUZUMAB *vs.* RUCAPARIB *vs.* surveillance	-Maintenance treatment, locally advanced/ metastatic HER2+/ -EG, adenocarcinoma (after 1st line chemo)	-PFS (RECIST)	-PFR -OS, ORR (RECIST) -% AE -PFS, PFR, OS, ORR according to PD-L1 immunohistochemical status	770	Pending
Durvalumab	NCT02264678	I / II	Recruiting	-AZD6738 ± Chemo/ OLAPARIB/DURVA	-Advanced malignancies (including GC)	-Safety, tolerability (AE, SAE)	-Pharmacodynamics, biomarker changes -Serum concentration of drug, half-life, etc. -BOR, ORR, % change in tumor size	250	Pending

NIVO: Nivolumab; IPI: Ipilimumab; TREME: Tremelimumab; DURVA: Durvalumab; PEMBRO: Pembrolizumab; AVE: Avelumab; TRAS: Trastuzumab; Chemo: Chemotherapy; AE: Adverse events; SAE: Serious adverse events; DLT: Dose limiting toxicities; DOR: Duration of response; OS: Overall survival; QOL: Quality of life; PFS: Progression free survival; ir-PFS: Immune-related progression free disease; PFR: Progression-free rate; ORR: Objective response rate; RR: Response rate; pathCR: Pathologic complete response; RFS: Relapse-free survival; DR: Disease response; DDC: Duration disease control; BOR: Best objective response; DCR: Disease control rate; PD: Progressive disease; CR: Complete response; PR: Partial response; SD: Stable disease; TTR: Time to response; NET: Neuroendocrine tumors; SqCC: Squamous cell carcinoma; GIST: Gastrointestinal stromal tumors; EBV: Epstein Barr virus; N/A: Not applicable.

shorter, and the toxicities were higher in the ipilimumab group, with similar OS for both groups[218].

(2) PD-1 and PD-L1 checkpoint inhibitors
Anti-PD-1 antibodies - Nivolumab
Nivolumab is a humanized immunoglobulin G4 monoclonal antibody (mAb) that targets PD-1 and has shown efficacy in various cancer types. The FDA approved nivolumab for the treatment of recurrent/metastatic melanoma, metastatic non-small cell lung carcinoma/recurrent or metastatic squamous cell carcinoma of the head and neck (after progression on platinum-based chemotherapy), advanced renal cell carcinoma after antiangiogenic treatment, and Hodgkin lymphoma that has relapsed/progressed after autologous hematopoietic stem cell transplantation.

The phase I / II CheckMate-032 clinical trial analyzed the safety and efficacy of nivolumab as monotherapy or combined with ipilimumab in heavily pretreated patients with advanced/metastatic solid cancers, including gastric tumors (NCT01928394)[219]. The partial results assessing 160 patients included in three different arms [nivolumab 3 mg/kg (N3), nivolumab 1 mg/kg plus ipilimumab 3 mg/kg (N1+I3), and nivolumab 3 mg/kg + ipilimumab 1 mg/kg (N3+I1)] showed more frequent toxicities in the N1+I3 group. The most commonly encountered adverse events in all the subsets were fatigue, pruritus, nausea, diarrhea and loss of appetite; thyroid damage was more frequent in the N1+I3 group. The overall objective response rate (ORR) was 16%, which was the highest in the N1+I3 group (26%); the disease control rate (DCR) was 38%. The median OS was longest in the N1+I3 arm (6.9 mo), followed by the N3 (5.0 mo) and the N3+I1 arms (4.8 mo). The response rates for patients with PD-L1+ tumors (> 1% of immunohistochemically-positive cells), both in patients treated with nivolumab monotherapy and combined treatment N1+I3 and N3+I1, were superior compared to patients with negative tumors (19% *vs* 12%, 40% *vs* 22% and 23% *vs* 0%, respectively).

Because of the promising results obtained using N1+I3 combination, the CheckMate-649 phase III clinical trial (NCT02872116) was initiated. In this study, the clinicians plan to investigate 1349 patients with naive advanced or metastatic gastric/gastroesophageal junction (GEJ) cancer, with both positive/negative PD-L1 expression to receive

first line nivolumab plus ipilimumab or nivolumab plus standard chemotherapy (XELOX or FOLFOX) *vs* standard chemotherapy (XELOX or FOLFOX), having as a primary endpoint the OS in patients with PD-L1[+] tumors[220]. The data from the double-blinded, randomized, multicentric phase Ⅲ trial ONO-12 (ATTRACTION 2) were presented at the ASCO Gastrointestinal Cancers Symposium 2017, demonstrating the benefits of nivolumab as a salvage treatment (third or later line) in patients with advanced unresectable or recurrent gastric or gastroesophageal junction cancer, either PD-1/L1+ or - *vs* placebo (NCT022673430). It was the first time that a prolonged OS was obtained for patients with heavily pretreated tumors using PD-1 inhibition[221]. There were 493 patients randomly assigned (2:1) to receive nivolumab (*n* = 330) or placebo (*n* = 163). The median OS was significantly increased with nivolumab *vs* placebo (5.26 mo *vs* 4.14 mo with placebo). The risk of death was lower in the nivolumab group *vs* placebo group (HR = 0.63; *P* < 0.0001); 68.5% of the patients in the nivolumab group died *vs* 86.5% in the placebo group. Additionally, nivolumab treatment was associated with a significantly better OS rates at 6 and 12 mo compared to placebo (46.1% *vs* 34.7% and 26.2% *vs* 10.9%), Nivolumab treatment led to a longer median PFS (1.61 mo *vs* 1.45 mo) and higher ORR than placebo (11.2% with nivolumab *vs* 0%). Among the approximately 40% (*n* = 192) of patients with tumor samples, 12.3% in the nivolumab group and 16.1% in the placebo group had PD-L1 positive tumors. The analysis of PD-L1 expression status showed that median OS in patients with PD-L1[+] tumors was 5.22 mo in the nivolumab group and 3.83 mo in the placebo group (HR = 0.51). In patients with PD-L1[-] tumors, median OS was 6.05 mo in the nivolumab group, and 4.19 in the placebo group (HR = 0.72). Nivolumab resulted in an absolute survival benefit of 1.1 mo in median OS *vs* placebo. Nivolumab led to durable OS benefit that was sustained beyond one year *vs* placebo in heavily pretreated gastric cancer patients, regardless of PD-L1 expression status[222]. Because of the survival benefit demonstrated by the ATTRACTION-2 trial in this subset of difficult-to-treat gastric cancer patients, the approval of nivolumab in Japan was granted as a new treatment option that is beneficial for heavily pretreated advanced gastric cancer.

Anti-PD-1 antibodies - Pembrolizumab
Pembrolizumab (formerly MK-3475, trade name Keytruda) is a humanized IgG4 isotype antibody that targets the PD-1 receptors on lymphocytes. It has FDA approval for the treatment of unresectable or metastatic melanoma, metastatic non-small cell lung cancer (NSCLC) (certain situations), metastatic non-squamous NSCLC (PDL1^{+}/[-]), as a second-line treatment for head and neck squamous cell carcinoma (HNSCC), after platinum-based chemotherapy, and for the treatment of adult/pediatric patients with refractory classic Hodgkin's lymphoma.

The large multi-cohort, multicenter, nonrandomized, open-label phase Ib KEYNOTE-012 first demonstrated the efficacy of pembrolizumab as monotherapy (administered in a dose of 10 mg/kg every 2 wk or a 200 mg fixed dose every 3 wk) in PD-L1[+] recurrent or metastatic gastric or gastroesophageal cancer (NCT01848834)[25]. Of all the patients assessed, 39 patients (40%) had PD-L1[+] tumors, most of them being heavily pretreated patients. Treatment with single-agent pembrolizumab determined partial response (PR) in 22% and sustained disease (SD) in 13% of patients. The median PFS was 1.9 mo, with a 6 mo PFS of 26% and a median OS of 11.4 mo. The most common adverse events included loss of appetite, fatigue, pruritus, arthralgia and hypothyroidism. Pembrolizumab was well-tolerated, with 13% of patients developing grade 3-4 toxicity. It is important to stress that approximately 60% of patients enrolled in this trial have previously received more than three lines of chemotherapy, representing a group of patients without any therapeutic response demonstrated by other studies. Almost two-thirds of the tumors revealed genomic profiling of a microsatellite-instability high (MSI-H) status. This genomic status correlates with high tumor mutational burden, and patients with MSI-H cancers, colorectal and non-colorectal, have developed encouraging responses to anti-PD1 therapy in various solid tumors[23]. This aspect remains to be established if present in gastroesophageal cancers. Additionally, in this study, tumor response correlated with an increased interferon-γ gene expression[223].

With the goal of improving immune treatment efficacy and having the knowledge that chemotherapy is capable of promoting immunogenic cell death[224], PD-1 inhibitors were associated with standard chemotherapy, which is a combined regimen proven to increase response rates in lung cancer[225]. The KEYNOTE-059 phase Ⅱ clinical trial included 259 patients with advanced gastric or gastroesophageal cancer. Cohort 1 assessed the efficacy and safety of pembrolizumab monotherapy in patients with previously treated advanced gastric cancer. Approximately half the patients who enrolled in cohort 1 had received two or more prior treatments for metastatic cancer, whereas others received three or more prior therapies. Among 259 patients, 148 (57.1%) were PD-L1[+] (by immunohistochemistry). Pembrolizumab elicited sustained ORR in 30 of 259 patients (11.6%) and complete response in 2.3%. These responses were observed irrespective of PD-L1 expression. The ORR was higher in patients with PD-L1[+] *vs* PD-L1[-] tumors, 23 of 148 (15.5%) *vs* 7 of 109 (6.4%), respectively. A T cell-inflamed gene expression profiling score was developed in this study, demonstrated to be significantly associated with pembrolizumab response; also, a significant non-linear association was found between this score and PD-L1 expression. Of 174 patients (67.2%) assessed for MSI, seven patients (4.0%) had samples that were MSI-high. It was observed a higher ORR in patients with MSI-high tumors than in patients with non-MSI-high tumors (57.1% *vs* 9.0%). However, prevalence of MSI high tumors was very low in this population (4%), and most responses were observed in non-MSI-high

patients. Thus, in cohort 1, pembrolizumab monotherapy showed good responses and manageable toxicities after ≥ 2 prior lines of treatment[226,227]; these results led to an accelerated FDA approval of pembrolizumab for PD-L1[+] advanced gastric cancer patients as third-line treatment. In cohort 2, the enrollees were HER2^{-} naïve patients with advanced gastric/gastroesophageal junction adenocarcinoma, who receive pembrolizumab associated with combined chemotherapy (5-fluorouracil/capecitabine plus cisplatin) for six cycles, and subsequent maintenance treatment with pembrolizumab plus 5-FU/capecitabine for a maximum period of two years/until disease progression[228]. The preliminary data showed that 94% of patients have experienced adverse events (*e.g.*, anorexia, nausea, neutropenia); of these patients, two thirds developed grade 3-4 toxicities. The results of the study proved manageable adverse event profiles for patients receiving combined therapeutic schemes, with none of the patients discontinuing the treatment. The data showed an ORR was 60% and 68.8% in PD-L1[+] patients[229].

The KEYNOTE-028 phase I b study evaluated the role of pembrolizumab, administered in up to two years or until progression, in PD-L1[+] advanced solid tumors, including esophageal and gastroesophageal junction cancers (adenocarcinoma and squamous cell cancer), with most of them having at least two prior lines of chemotherapy[230]. The interim data on 23 patients showed an ORR of 30%, with 6- and 12-mo PFS rates of 30.4% and 21.7%, respectively. The response rates were better for adenocarcinomas.

An ongoing phase II study is assessing the efficiency of pembrolizumab monotherapy, along with the analysis of immune-related gene profiles and PD-L1 expression as biomarkers for treatment response in patients with advanced cancer of the esophagus or gastroesophageal junction (adenocarcinoma or squamous cell carcinoma) (KEYNOTE-180, NCT02559687)[231], progressing on standard chemotherapy.

KEYNOTE-061 is an ongoing phase III open-label clinical trial comparing pembrolizumab versus paclitaxel in the second-line setting for patients with advanced gastric or gastroesophageal cancer (progression after first-line therapy with a platinum plus fluoropyrimidine combination) (NCT02370498)[232]. The treatment administration continues until the disease progression or the occurrence of severe toxicities; the primary endpoints are PFS and OS in the PD-L1[+] population. The study randomized 592 patients to receive pembrolizumab or standard-dose paclitaxel. PD-L1 positivity was encountered in 395/592 patients enrolled. Median OS was 9.1 mo with pembrolizumab *vs* 8.3 mo with paclitaxel (HR = 0.82, *P* = 0.042); 12-mo OS rates in pembrolizumab group compared with paclitaxel group were 39.8% *vs* 27.1%, and 18-mo rates were 25.7% *vs* 14.8%. There was no difference in PFS or ORR, but pembrolizumab responses proved more durable, and the treatment effect was more prominent in patients with ECOG PS 0 (HR = 0.69), gastroesophageal junction tumors

(HR 0.61) and with increasing PD-L1 expression. The safety profile observed in KEYNOTE-061 was consistent with that observed in previously reported studies of pembrolizumab, this drug demonstrating a better safety profile than paclitaxel. The agent reduced the risk of death by 18% *vs* paclitaxel in patients with previously treated gastric cancer and PD-L1[+], although this difference did not achieve statistical significance[233].

Furthermore, the ongoing phase III KEYNOTE-062 study is randomizing PD-L1[+]/HER2^{-} advanced, metastatic gastric/GEJ adenocarcinoma patients to receive as a first-line regimen either pembrolizumab or pembrolizumab, associated with fluorouracil and cisplatin (NCT02494583)[234]. The primary endpoints are represented by OS and PFS.

A phase III study (KEYNOTE-181) aims to include approximately 600 patients with previously treated advanced adenocarcinoma or squamous cell carcinoma of the esophagus or gastroesophageal junction to receive either pembrolizumab or monochemotherapy (paclitaxel, docetaxel, or irinotecan) (NCT02564263)[235].

Anti-PD-L1 antibodies - Avelumab

Avelumab (MSB0010718C, trade name Bavencio) represents a fully human anti-PD-L1 IgG1 antibody which is approved by the FDA for the treatment of metastatic Merkel cell carcinoma (patients older than 12 years) and locally advanced/metastatic urothelial carcinoma under progression, after platinum-containing treatment (2017).

Avelumab is currently assessed in JAVELIN program (NCT01772004)[236]. Patients received 10 mg/kg of avelumab every two wks as second-line treatment. For Japanese gastric cancer patients, the obtained ORR was 15%, with 43.3% of patients presenting PFS at 12 wk[237]. In this context, phase I b trials are currently investigating avelumab in patients with advanced gastric/gastroesophageal junction cancers. The results, presented at the American Society of Clinical Oncology (ASCO) 2016 meeting, showed that the most encountered adverse events were infusion-related reactions and fatigue. In the first context (patients who had at least one prior therapy), the ORR, DCR, and median PFS were 9.7%, 29.0%, and 6.0 wk, respectively, whereas in the second setting (patients who received avelumab as first-line switch maintenance after chemotherapy), they were 9%, 57.3%, and 12.0 wk, respectively. ORR in the PD-L1[+] tumors was higher *vs* PD-L1[-] tumors (18.2% *vs* 9.1% for the third-line treatment group and 10% *vs* 3.1% for the maintenance group). Starting from these data, there are two phase III ongoing studies assessing avelumab in the treatment of gastric cancer patients. One study is comparing avelumab as a third-line of treatment to best supportive care (phase III JAVELIN Gastric 300 trial) (NCT02625623), while another is assessing patients receiving avelumab as a switch maintenance after chemotherapy with 5-FU or capecitabine plus oxaliplatin (phase III JAVELIN Gastric 100 study) (NCT02625610)[238]. The Javelin Gastric 300 trial enrolled 371 patients from 147 sites in Asia,

Figure 3 Therapeutic algorithm in unresectable locally advanced, recurrent or metastatic gastric cancer. HER2: Human epidermal growth factor receptor 2; CX: Cisplatin and capecitabine; CF: Cisplatin and fluorouracil; MSI: Microsatellite instability; dMMR: Deficient mismatch repair gene; PD-L1: Programmed death ligand-1.

Australia, Europe, North America and South America. Recently, the companies stated that the pivotal phase Ⅲ Javelin trial investigating avelumab as third-line treatment for patients with unresectable, recurrent or metastatic gastric cancer did not meet its pre-specified primary endpoint of superior OS *vs* chemotherapy. It represents the first trial of a checkpoint *inhibitor vs* an active chemotherapy comparator rather than placebo in this hard-to-treat patient population. The safety profile was consistent with that observed in previously reported studies of avelumab. The JAVELIN Gastric 300 data will be further evaluated in an effort to better understand the results.

Anti-PD-L1 antibodies - Durvalumab (MEDI4736)
Durvalumab represents an engineered human anti-PD-L1 IgG1 antibody that prevents PD-L1 binding to PD-1 and CD80. It has received FDA approval for previously treated advanced bladder carcinoma and unresectable stage Ⅲ NSCLC.

A phase Ⅰ study assessed the efficiency of ad-

ministering durvalumab in doses up to 10 mg/kg intravenously every two weeks for up to one year in patients with solid tumors, including gastric cancer (NCT01693562)[239]. Fatigue, nausea, vomiting, rash and pyrexia were the most frequent adverse events. An ORR of 25% was obtained in patients with gastric tumors. Additionally, two patients with heavily pretreated tumors surpassed the current median PFS that was obtained with standard treatments.

An ongoing phase Ⅰ B/Ⅱ study is investigating patients with recurrent/metastatic gastric/gastroesophageal junction adenocarcinomas for monotherapy with durvalumab, tremelimumab, or a combination of durvalumab and tremelimumab (anti-CTLA-4) in second- or third-line setting[240].

An algorithm of current treatment in unresectable locally advanced, recurrent or metastatic gastric cancer is presented in Figure 3.

Checkpoint inhibitors in the adjuvant /neoadjuvant setting (stage Ⅱ or Ⅲ tumors): Because multiple

lines of standard chemotherapy may harm the immune system, and immune biomarkers may be stimulated by previous chemoradiation, it was suggested that addition of checkpoint inhibition in the adjuvant or even neoadjuvant setting in earlier stages of the disease may induce a significantly higher tumor response rate[241]. It has been demonstrated that after chemoradiation neoadjuvant therapy, there is a higher number of TILs that are agglomerated in perivascular areas and arranged in lymphoid-like structures; these are features that favor the tumor response to immunotherapy[58].

A randomized phase Ⅲ study (CheckMate-577) NCT02743494 is assessing the role of adjuvant nivolumab for patients with resected esophageal/gastroesophageal cancer. Investigators aim to enroll approximately 760 patients to receive either nivolumab or placebo. The primary endpoints are OS and DFS[242]. Moreover, a phase Ⅰ study is currently investigating nivolumab combined with an anti-CCR4 (mogamulizumab) in the preoperative setting (NCT02946671)[243].

The combination of nivolumab and ipilimumab is going to be assessed in the adjuvant treatment of gastric/gastroesophageal junction adenocarcinomas with a high risk of recurrence - a phase Ⅱ study (NCT03443856) (VESTIGE)[244].

Several ongoing phase Ⅰ/Ⅱ clinical trials are assessing the safety and efficacy of neoadjuvant treatment using different PD-1/PD-L1 checkpoint inhibitors, administered either concomitantly or sequentially with neoadjuvant chemoradiation. For example, a phase Ⅰ trial is assessing neoadjuvant administration of nivolumab and ipilimumab in stage Ⅱ/Ⅲ patients (NCT03044613)[245]; another Ⅰ b phase clinical trial is assessing pembrolizumab and chemoradiation in the neoadjuvant setting of locally advanced esogastric tumors (NCT03064490/PROCEED)[246]. Several Ⅱ and Ⅲ phase ongoing studies are investigating the efficacy of pembrolizumab in association with chemotherapy as neoadjuvant/adjuvant treatment in gastric cancer patients[247-249]. Additionally, a phase Ⅱ study is evaluating the results of administering avelumab plus chemotherapy (FLOT) in the perioperative setting[250].

FUTURE PERSPECTIVES

The synergistic action of the PD-1/PD-L1 checkpoint inhibitors and the vascular endothelial growth factor (VEGF)/vascular endothelial growth factor receptor (VEGFR) blockade was demonstrated by preclinical data[251]; the combined treatment induced an improved tumor response and better OS. This combination treatment assessed in phase Ⅰ/Ⅱ studies was associated with an acceptable safety profile [252,253].

A phase Ⅰa/Ⅰb study (NCT02443324)[254] is investigating the safety and efficacy of combining anti-PD-L1 (durvalumab) and anti-VEGFR2 antibodies (ramucirumab) in patients with refractory gastric/gastroesophageal junction tumors. The interim data of 40 patients showed a 45% DCR and a median PFS of 2.60 mo. The most frequently encountered toxicities included fatigue,

infusion-related reaction, loss of appetite, pruritus, rash, and hypertension; 25% of patients had severe toxicities.

Additionally, the efficacy of associating nivolumab with ramucirumab in patients with advanced, unresectable gastric/gastroesophageal junction cancers is currently under investigation in a phase Ⅰ/Ⅱ study (NCT02999295)[255]. The clinical trial of nivolumab with paclitaxel and ramucirumab in patients with advanced, unresectable gastric cancer is ongoing under investigation in a phase Ⅰ/Ⅱ study (UMIN000025947)[256].

A phase Ⅱ study is currently recruiting patients with metastatic/recurrent gastric/gastroesophageal cancer to receive pembrolizumab plus lenvatinib mesylate (anti-VEGFR2 tyrosine kinase inhibitor) (NCT03413397)[257], whereas another phase Ⅰ/Ⅱ study is planning to combine avelumab with regorafenib (another anti-VEGFR2 tyrosine kinase inhibitor) in advanced gastric tumors (NCT03475953/REGOMUNE)[258].

Because HER2$^+$ tumors develop resistance to trastuzumab, studies have been evaluating a combination of PD-1 blockade plus anti-HER2 agents. Preclinical studies indicate that the HER2 blockade stimulates T cell activation and enhances interferon-γ secretion by NK cells and antibody-dependent cellular toxicity; therefore, it boosts the PD-1/L1 inhibitor efficacy[259].

The combination of pembrolizumab plus the anti-HER2 monoclonal antibody margetuximab in patients with advanced HER2$^+$ gastric cancers that are resistant to classical trastuzumab-based chemotherapy is currently under evaluation in a phase Ⅰb/Ⅱ dose-escalation trial (NCT02689284)[260].

Other promising options include dual immunotherapies, such as the administration of PD-1/PD-L1 checkpoint inhibitors combined with agents suppressing other immune checkpoints (*e.g.*, TIM3, LAG3) or T cell costimulatory antibodies (*e.g.*, GITR, OX40, and 4-1BB). Additionally, other directions investigate the combination of the PD-1/PD-L1 blockade with radiation and other cytotoxic and targeted treatments (PARP inhibitors, ATR serine/threonine protein kinase inhibitors, pegylated recombinant human hyaluronidase, anti-CEACAM1, arginase inhibitor INCB001158, FGFR inhibitors, immunotherapy using *Listeria* bacteria to activate an immune response against specific tumor-associated antigens, claudiximab, *etc.*), and enzymatic inhibitors such as IDO-1[211].

Therapeutic intervention upon IDO1-mediated immune suppression involves inhibition of the catalytic activity of IDO1. Preliminary data from the ongoing studies investigating dual inhibition of both PD-1 and IDO1 in solid tumors are promising, showing an increased efficiency of the checkpoint blockade[261,262]. Currently, there are two studies of phase Ⅰ/Ⅱ (NCT02178722/KEYNOTE-037/ECHO-202)[263] and Ⅱ (NCT03196232)[264], respectively investigating the IDO1 inhibitor epacadostat in combination with pembrolizumab in gastric tumors.

Additionally, CDC20 encoding cell division cycle protein 20 homologue[265], as well as PLK1 and TTK,

which represent checkpoints in mitosis, may be used as therapeutic targets[266]. PLK1 is upregulated in tumors, and data reported from both preclinical and clinical studies suggest encouraging benefits from PLK-1 inhibition[267]. Maternal embryonic leucine zipper kinase (MELK) decreases the apoptosis of tumor cells; therefore, additional MELK inhibition may also be offered in PD-L1+ cancers[268].

The negative checkpoint regulator VISTA represents a V-domain Ig suppressor of T cell activation (VISTA), known as PD1 homolog, which belongs to the B7 family. VISTA is mostly exhibited on hematopoietic cells[269]. In murine tumor models, anti-VISTA monoclonal antibodies activated intratumoral T cells, enhancing antitumor immunity. Moreover, the combined VISTA/PD-1 blockade was proven efficient[270,271]. A phase I study, combining VISTA and PD-L1/PD-L2 inhibition in solid tumors (CA-170 molecule; NCT02812875), has been initiated[272]. Böger et al[273], investigated VISTA expression in 464 gastric cancer patients, with an increased expression associated with the intestinal type (VISTA is a regulator of differentiation), proximal gastric tumors, and KRAS- and PIK3CA mutant cancers. Additionally, a significantly higher VISTA expression in immune cells was noticed in association with PD-L1 expression and in EBV+ tumors. These observations might stress that a subset of tumors may use multiple checkpoint pathways to escape immunity and that the combined VISTA/PD-1 inhibition may be a promising novel cancer approach.

Another study is investigating nivolumab plus a matrix metalloproteinase 9 inhibitor (GS-5745 = andecaliximab) in patients with unresectable/recurrent gastric or gastroesophageal junction adenocarcinoma (NCT02864381)[274].

Due to the permanent development of novel immunotherapeutic agents, classical trials do not have the ability to appropriately assess all possible drug combinations. In this regard, FRACTION (Fast Real-time Assessment of Combination Therapies in Immuno-Oncology) is a new clinical trial program with a dynamic design that provides the possibility both for the inclusion of new immunotherapeutic combinations, as well as exclusion of ineffective ones[275].

Cancer radiotherapy may seldom exhibit the clinical phenomenon of the abscopal response, meaning that no irradiated metastases diminish after radiation to the primary tumor. Data from preclinical trials highlighted that radiotherapy plus PD-1/PD-L1 blockade exhibited synergistic anticancer effects. Currently, trials on gastric cancer patients are investigating the efficiency of pembrolizumab plus palliative radiotherapy (metastatic tumors), and additional neoadjuvant chemoradiotherapy (resectable tumors) (NCT02730546)[276], respectively. A phase I/II trial is also investigating nivolumab+ radiotherapy in unresectable, recurrent gastric cancer (as third-line treatment) (NCT03453164) (CIRCUIT)[277].

PREDICTIVE BIOMARKERS AND GENETIC PROFILES

The combination of immune checkpoint inhibitors with other types of immunotherapies, targeted agents, radio- or chemotherapies, seems to generate promising results against gastrointestinal cancers but at the expense of the occurrence of immune-related side effects, some of them potentially fatal[278,279]. Therefore, identification of prognostic biomarkers and genetic profiles to define subgroups of gastric cancer patients who are most likely to respond to specific immunotherapeutic regimens is an urgent need[280].

In this regard, an ongoing observational study NCT02951091 (Biomarker - integrated Umbrella)[281] is investigating different molecular cohorts in oncologic patients, the cases of PD-L1+, MSI-H, and EBV+ advanced gastric cancers being assigned to receive either nivolumab or other agents, such as AFATINIB (EGFR tyrosine kinase inhibitor or GSK2636771 (PI3K beta inhibitor) plus PACLITAXEL). A phase II study compares nivolumab with other novel agents, according to genetic testing, in gastric cancer patients with mismatch repair deficiency (loss of MLH1/ MLH2) (NCT02465060 - The MATCH screening trial)[282].

CONCLUSION

Because of the well-known heterogeneity of tumors, it is essential to assess the particular molecular biology of different subtypes of gastric cancers that are associated with different clinico-biologic parameters and prognosis to identify innovative treatment approaches that will improve current results in gastric cancer. Modern treatments, such as the promising immunotherapy, should be applied in this way to selected patients who have been proven to have the best response to a specific therapy (individualized treatment). In this context, it is also mandatory to discover prognostic tumor markers and predictive biomarkers for treatment response.

REFERENCES

1   **Ferlay J**, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. Estimated incidence, mortality and prevalence worldwide in 2012. 2013. [Internet]. Lyon, France: International Agency for Research on Cancer. Accessed May 4, 2018 Available from: URL: http://globocan.iarc.fr/Default.aspx

2   **Waddell T**, Verheij M, Allum W, Cunningham D, Cervantes A, Arnold D; European Society for Medical Oncology (ESMO); European Society of Surgical Oncology (ESSO); European Society of Radiotherapy and Oncology (ESTRO). Gastric cancer: ESMO-ESSO-ESTRO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2013; **24** Suppl 6: vi57-vi63 [PMID: 24078663 DOI: 10.1093/annonc/mdt344]

3   **Okines AF**, Norman AR, McCloud P, Kang YK, Cunningham D. Meta-analysis of the REAL-2 and ML17032 trials: evaluating capecitabine-based combination chemotherapy and infused 5-fluorouracil-based combination chemotherapy for the treatment of advanced oesophago-gastric cancer. *Ann Oncol* 2009; **20**: 1529-1534 [PMID: 19474114 DOI: 10.1093/annonc/mdp047]

4   **Bang YJ**, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Rüschoff J, Kang YK; ToGA Trial Investigators. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet* 2010; **376**: 687-697 [PMID: 20728210 DOI: 10.1016/S0140-6736(10)61121-X]

5   **Fuchs CS**, Tomasek J, Yong CJ, Dumitru F, Passalacqua R, Goswami C, Safran H, Dos Santos LV, Aprile G, Ferry DR, Melichar B, Tehfe M, Topuzov E, Zalcberg JR, Chau I, Campbell W, Sivanandan C, Pikiel J, Koshiji M, Hsu Y, Liepa AM, Gao L, Schwartz JD, Tabernero J; REGARD Trial Investigators. Ramucirumab monotherapy for previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (REGARD): an international, randomised, multicentre, placebo-controlled, phase 3 trial. *Lancet* 2014; **383**: 31-39 [PMID: 24094768 DOI: 10.1016/S0140-6736(13)61719-5]

6   **Wilke H**, Muro K, Van Cutsem E, Oh SC, Bodoky G, Shimada Y, Hironaka S, Sugimoto N, Lipatov O, Kim TY, Cunningham D, Rougier P, Komatsu Y, Ajani J, Emig M, Carlesi R, Ferry D, Chandrawansa K, Schwartz JD, Ohtsu A; RAINBOW Study Group. Ramucirumab plus paclitaxel versus placebo plus paclitaxel in patients with previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (RAINBOW): a double-blind, randomised phase 3 trial. *Lancet Oncol* 2014; **15**: 1224-1235 [PMID: 25240821 DOI: 10.1016/S1470-2045(14)70420-6]

7   **Hodi FS**, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbé C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010; **363**: 711-723 [PMID: 20525992 DOI: 10.1056/NEJMoa1003466]

8   **Robert C**, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, Lebbe C, Baurain JF, Testori A, Grob JJ, Davidson N, Richards J, Maio M, Hauschild A, Miller WH Jr, Gascon P, Lotem M, Harmankaya K, Ibrahim R, Francis S, Chen TT, Humphrey R, Hoos A, Wolchok JD. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* 2011; **364**: 2517-2526 [PMID: 21639810 DOI: 10.1056/NEJMoa1104621]

9   **Lyons TG**, Ku GY. Systemic therapy for esophagogastric cancer: immune checkpoint inhibition. *Chin Clin Oncol* 2017; **6**: 53 [PMID: 29129093 DOI: 10.21037/cco.2017.09.03]

10   **Lauwers GY,** Carneiro F, Graham DY. Gastric carcinoma. In: Bowman FT, Carneiro F, Hruban RH, eds. Classification of Tumours of the Digestive System. Lyon: IARC, 2010

11   **Lauren P**. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. *Acta Pathol Microbiol Scand* 1965; **64**: 31-49 [PMID: 14320675 DOI: 10.1111/apm.1965.64.1.31]

12   **Garattini SK**, Basile D, Cattaneo M, Fanotto V, Ongaro E, Bonotto M, Negri FV, Berenato R, Ermacora P, Cardellino GG, Giovannoni M, Pella N, Scartozzi M, Antonuzzo L, Silvestris N, Fasola G, Aprile G. Molecular classifications of gastric cancers: Novel insights and possible future applications. *World J Gastrointest Oncol* 2017; **9**: 194-208 [PMID: 28567184 DOI: 10.4251/wjgo.v9.i5.194]

13   **Lei Z**, Tan IB, Das K, Deng N, Zouridis H, Pattison S, Chua C, Feng Z, Guan YK, Ooi CH, Ivanova T, Zhang S, Lee M, Wu J, Ngo A, Manesh S, Tan E, Teh BT, So JB, Goh LK, Boussioutas A, Lim TK, Flotow H, Tan P, Rozen SG. Identification of molecular subtypes of gastric cancer with different responses to PI3-kinase inhibitors and 5-fluorouracil. *Gastroenterology* 2013; **145**: 554-565 [PMID: 23684942 DOI: 10.1053/j.gastro.2013.05.010]

14   **Lim B**, Kim JH, Kim M, Kim SY. Genomic and epigenomic heterogeneity in molecular subtypes of gastric cancer. *World J Gastroenterol* 2016; **22**: 1190-1201 [PMID: 26811657 DOI: 10.3748/wjg.v22.i3.1190]

15   **Aprile G**, Giampieri R, Bonotto M, Bittoni A, Ongaro E, Cardellino GG, Graziano F, Giuliani F, Fasola G, Cascinu S, Scartozzi M. The challenge of targeted therapies for gastric cancer patients: the beginning of a long journey. *Expert Opin Investig Drugs* 2014; **23**: 925-942 [PMID: 24806575 DOI: 10.1517/13543784.2014.912631]

16   **Chen T**, Xu XY, Zhou PH. Emerging molecular classifications and therapeutic implications for gastric cancer. *Chin J Cancer* 2016; **35**: 49 [PMID: 27233623 DOI: 10.1186/s40880-016-0111-5]

17   **Tan P**, Yeoh KG. Genetics and Molecular Pathogenesis of Gastric Adenocarcinoma. *Gastroenterology* 2015; **149**: 1153-1162.e3

[PMID: 26073375 DOI: 10.1053/j.gastro.2015.05.059]

18   **Pedrazzani C**, Corso G, Velho S, Leite M, Pascale V, Bettarini F, Marrelli D, Seruca R, Roviello F. Evidence of tumor microsatellite instability in gastric cancer with familial aggregation. *Fam Cancer* 2009; **8**: 215-220 [PMID: 19152022 DOI: 10.1007/s10689-008-9231-7]

19   **Velho S**, Fernandes MS, Leite M, Figueiredo C, Seruca R. Causes and consequences of microsatellite instability in gastric carcinogenesis. *World J Gastroenterol* 2014; **20**: 16433-16442 [PMID: 25469011 DOI: 10.3748/wjg.v20.i44.16433]

20   **Chung DC**, Rustgi AK. DNA mismatch repair and cancer. *Gastroenterology* 1995; **109**: 1685-1699 [PMID: 7557155 DOI: 10.1016/0016-5085(95)90660-6]

21   **Zhu L**, Li Z, Wang Y, Zhang C, Liu Y, Qu X. Microsatellite instability and survival in gastric cancer: A systematic review and meta-analysis. *Mol Clin Oncol* 2015; **3**: 699-705 [PMID: 26137290 DOI: 10.3892/mco.2015.506]

22   **Topalian SL**, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 2012; **366**: 2443-2454 [PMID: 22658127 DOI: 10.1056/NEJMoa1200690]

23   **Le DT**, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, Skora AD, Luber BS, Azad NS, Laheru D, Biedrzycki B, Donehower RC, Zaheer A, Fisher GA, Crocenzi TS, Lee JJ, Duffy SM, Goldberg RM, de la Chapelle A, Koshiji M, Bhaijee F, Huebner T, Hruban RH, Wood LD, Cuka N, Pardoll DM, Papadopoulos N, Kinzler KW, Zhou S, Cornish TC, Taube JM, Anders RA, Eshleman JR, Vogelstein B, Diaz LA Jr. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med* 2015; **372**: 2509-2520 [PMID: 26028255 DOI: 10.1056/NEJMoa1500596]

24   **Schwitalle Y**, Kloor M, Eiermann S, Linnebacher M, Kienle P, Knaebel HP, Tariverdian M, Benner A, von Knebel Doeberitz M. Immune response against frameshift-induced neopeptides in HNPCC patients and healthy HNPCC mutation carriers. *Gastroenterology* 2008; **134**: 988-997 [PMID: 18395080 DOI: 10.1053/j.gastro.2008.01.015]

25   **Muro K**, Chung HC, Shankaran V, Geva R, Catenacci D, Gupta S, Eder JP, Golan T, Le DT, Burtness B, McRee AJ, Lin CC, Pathiraja K, Lunceford J, Emancipator K, Juco J, Koshiji M, Bang YJ. Pembrolizumab for patients with PD-L1-positive advanced gastric cancer (KEYNOTE-012): a multicentre, open-label, phase 1b trial. *Lancet Oncol* 2016; **17**: 717-726 [PMID: 27157491 DOI: 10.1016/S1470-2045(16)00175-3]

26   **Bristol-Myers Squibb**. A Study of Nivolumab and Nivolumab Plus Ipilimumab in Recurrent and Metastatic Colon Cancer (CheckMate 142). [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT02060188 ClinicalTrials.gov Identifier: NCT02060188

27   **Buckowitz A**, Knaebel HP, Benner A, Bläker H, Gebert J, Kienle P, von Knebel Doeberitz M, Kloor M. Microsatellite instability in colorectal cancer is associated with local lymphocyte infiltration and low frequency of distant metastases. *Br J Cancer* 2005; **92**: 1746-1753 [PMID: 15856045 DOI: 10.1038/sj.bjc.6602534]

28   **Cancer Genome Atlas Research Network**. Comprehensive molecular characterization of gastric adenocarcinoma. *Nature* 2014; **513**: 202-209 [PMID: 25079317 DOI: 10.1038/nature13480]

29   **Murphy G**, Pfeiffer R, Camargo MC, Rabkin CS. Meta-analysis shows that prevalence of Epstein-Barr virus-positive gastric cancer differs based on sex and anatomic location. *Gastroenterology* 2009; **137**: 824-833 [PMID: 19445939 DOI: 10.1053/j.gastro.2009.05.001]

30   **Camargo MC**, Murphy G, Koriyama C, Pfeiffer RM, Kim WH, Herrera-Goepfert R, Corvalan AH, Carrascal E, Abdirad A, Anwar M, Hao Z, Kattoor J, Yoshiwara-Wakabayashi E, Eizuru Y, Rabkin CS, Akiba S. Determinants of Epstein-Barr virus-positive gastric cancer: an international pooled analysis. *Br J Cancer* 2011; **105**: 38-43

[PMID: 21654677 DOI: 10.1038/bjc.2011.215]

31   **Kim SY**, Park C, Kim HJ, Park J, Hwang J, Kim JI, Choi MG, Kim S, Kim KM, Kang MS. Deregulation of immune response genes in patients with Epstein-Barr virus-associated gastric cancer and outcomes. *Gastroenterology* 2015; **148**: 137-147.e9 [PMID: 25254613 DOI: 10.1053/j.gastro.2014.09.020]

32   **Derks S**, Liao X, Chiaravalli AM, Xu X, Camargo MC, Solcia E, Sessa F, Fleitas T, Freeman GJ, Rodig SJ, Rabkin CS, Bass AJ. Abundant PD-L1 expression in Epstein-Barr Virus-infected gastric cancers. *Oncotarget* 2016; **7**: 32925-32932 [PMID: 27147580 DOI: 10.18632/oncotarget.9076]

33   **Cristescu R**, Lee J, Nebozhyn M, Kim KM, Ting JC, Wong SS, Liu J, Yue YG, Wang J, Yu K, Ye XS, Do IG, Liu S, Gong L, Fu J, Jin JG, Choi MG, Sohn TS, Lee JH, Bae JM, Kim ST, Park SH, Sohn I, Jung SH, Tan P, Chen R, Hardwick J, Kang WK, Ayers M, Hongyue D, Reinhard C, Loboda A, Kim S, Aggarwal A. Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. *Nat Med* 2015; **21**: 449-456 [PMID: 25894828 DOI: 10.1038/nm.3850]

34   **Yingyan Y**. A new molecular classification of gastric cancer proposed by Asian Cancer Research Group (ACRG). *Transl Gastrointest Cancer* 2016; **5**: 55-57 [DOI: 10.3978/j.issn.2224-4778.2015.12.01]

35   **Burnet M**. Cancer; a biological approach. I. The processes of control. *Br Med J* 1957; **1**: 779-786 [PMID: 13404306 DOI: 10.1136/bmj.1.5022.779]

36   **Matsueda S**, Graham DY. Immunotherapy in gastric cancer. *World J Gastroenterol* 2014; **20**: 1657-1666 [PMID: 24587645 DOI: 10.3748/wjg.v20.i7.1657]

37   **Ferrara N**, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003; **9**: 669-676 [PMID: 12778165 DOI: 10.1038/nm0603-669]

38   **Kandulski A**, Malfertheiner P, Wex T. Role of regulatory T-cells in H. pylori-induced gastritis and gastric cancer. *Anticancer Res* 2010; **30**: 1093-1103 [PMID: 20530414]

39   **Haraguchi N**, Inoue H, Tanaka F, Mimori K, Utsunomiya T, Sasaki A, Mori M. Cancer stem cells in human gastrointestinal cancers. *Hum Cell* 2006; **19**: 24-29 [PMID: 16643604 DOI: 10.1111/j.1749-0774.2005.00004.x]

40   **Houghton J**, Wang TC. Helicobacter pylori and gastric cancer: a new paradigm for inflammation-associated epithelial cancers. *Gastroenterology* 2005; **128**: 1567-1578 [PMID: 15887152 DOI: 10.1053/j.gastro.2005.03.037]

41   **Lin R**, Ma H, Ding Z, Shi W, Qian W, Song J, Hou X. Bone marrow-derived mesenchymal stem cells favor the immunosuppressive T cells skewing in a Helicobacter pylori model of gastric cancer. *Stem Cells Dev* 2013; **22**: 2836-2848 [PMID: 23777268 DOI: 10.1089/scd.2013.0166]

42   **Procaccio L**, Schirripa M, Fassan M, Vecchione L, Bergamo F, Prete AA, Intini R, Manai C, Dadduzio V, Boscolo A, Zagonel V, Lonardi S. Immunotherapy in Gastrointestinal Cancers. *Biomed Res Int* 2017; **2017**: 4346576 [PMID: 28758114 DOI: 10.1155/2017/4346576]

43   **Topalian SL**, Drake CG, Pardoll DM. Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. *Curr Opin Immunol* 2012; **24**: 207-212 [PMID: 22236695 DOI: 10.1016/j.coi.2011.12.009]

44   **Blank C**, Gajewski TF, Mackensen A. Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: implications for tumor immunotherapy. *Cancer Immunol Immunother* 2005; **54**: 307-314 [PMID: 15599732 DOI: 10.1007/s00262-004-0593-x]

45   **Balch CM**, Riley LB, Bae YJ, Salmeron MA, Platsoucas CD, von Eschenbach A, Itoh K. Patterns of human tumor-infiltrating lymphocytes in 120 human cancers. *Arch Surg* 1990; **125**: 200-205 [PMID: 1689143 DOI: 10.1001/archsurg.1990.01410140078012]

46   **Zheng X,** Song X, Shao Y, Xu B, Chen L, Zhou Q, Hu W, Zhang D, Wu C, Tao M, Zhu Y, Jiang J. Prognostic role of tumor-infiltrating lymphocytes in gastric cancer: a meta-analysis. *Oncotarget* 2017; **8**: 57386-57398 [PMID: 28915679 DOI: 10.18632/oncotarget.18065]

47   **Chen DS**, Mellman I. Oncology meets immunology: the cancer-

immunity cycle. *Immunity* 2013; **39**: 1-10 [PMID: 23890059 DOI: 10.1016/j.immuni.2013.07.012]

48   **Kang BW**, Kim JG, Lee IH, Bae HI, Seo AN. Clinical significance of tumor-infiltrating lymphocytes for gastric cancer in the era of immunology. *World J Gastrointest Oncol* 2017; **9**: 293-299 [PMID: 28808502 DOI: 10.4251/wjgo.v9.i7.293]

49   **Schreiber RD**, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 2011; **331**: 1565-1570 [PMID: 21436444 DOI: 10.1126/science.1203486]

50   **Kang BW**, Seo AN, Yoon S, Bae HI, Jeon SW, Kwon OK, Chung HY, Yu W, Kang H, Kim JG. Prognostic value of tumor-infiltrating lymphocytes in Epstein-Barr virus-associated gastric cancer. *Ann Oncol* 2016; **27**: 494-501 [PMID: 26673353 DOI: 10.1093/annonc/mdv610]

51   **Ishigami S**, Natsugoe S, Tokuda K, Nakajo A, Che X, Iwashige H, Aridome K, Hokita S, Aikou T. Prognostic value of intratumoral natural killer cells in gastric carcinoma. *Cancer* 2000; **88**: 577-583 [PMID: 10649250 DOI: 10.1002/(SICI)1097-0142(20000201)88:3<577::AID-CNCR13>3.0.CO;2-V]

52   **Dirican A**, Ekinci N, Avci A, Akyol M, Alacacioglu A, Kucukzeybek Y, Somali I, Erten C, Demir L, Can A, Bayoglu IV, Koyuncu B, Ulger E, Tarhan MO. The effects of hematological parameters and tumor-infiltrating lymphocytes on prognosis in patients with gastric cancer. *Cancer Biomark* 2013; **13**: 11-20 [PMID: 23736017 DOI: 10.3233/CBM-130331]

53   **Dai C**, Geng R, Wang C, Wong A, Qing M, Hu J, Sun Y, Lo AW, Li J. Concordance of immune checkpoints within tumor immune contexture and their prognostic significance in gastric cancer. *Mol Oncol* 2016; **10**: 1551-1558 [PMID: 27720576 DOI: 10.1016/j.molonc.2016.09.004]

54   **Haas M**, Dimmler A, Hohenberger W, Grabenbauer GG, Niedobitek G, Distel LV. Stromal regulatory T-cells are associated with a favourable prognosis in gastric cancer of the cardia. *BMC Gastroenterol* 2009; **9**: 65 [PMID: 19732435 DOI: 10.1186/1471-230X-9-65]

55   **Kim KJ**, Lee KS, Cho HJ, Kim YH, Yang HK, Kim WH, Kang GH. Prognostic implications of tumor-infiltrating FoxP3^{+} regulatory T cells and CD8^{+} cytotoxic T cells in microsatellite-unstable gastric cancers. *Hum Pathol* 2014; **45**: 285-293 [PMID: 24331841 DOI: 10.1016/j.humpath.2013.09.004]

56   **Chiaravalli AM**, Feltri M, Bertolini V, Bagnoli E, Furlan D, Cerutti R, Novario R, Capella C. Intratumour T cells, their activation status and survival in gastric carcinomas characterised for microsatellite instability and Epstein-Barr virus infection. *Virchows Arch* 2006; **448**: 344-353 [PMID: 16261379 DOI: 10.1007/s00428-005-0066-4]

57   **Liu K**, Yang K, Wu B, Chen H, Chen X, Chen X, Jiang L, Ye F, He D, Lu Z, Xue L, Zhang W, Li Q, Zhou Z, Mo X, Hu J. Tumor-Infiltrating Immune Cells Are Associated With Prognosis of Gastric Cancer. *Medicine (Baltimore)* 2015; **94**: e1631 [PMID: 26426650 DOI: 10.1097/MD.0000000000001631]

58   **Thompson ED**, Zahurak M, Murphy A, Cornish T, Cuka N, Abdelfatah E, Yang S, Duncan M, Ahuja N, Taube JM, Anders RA, Kelly RJ. Patterns of PD-L1 expression and CD8 T cell infiltration in gastric adenocarcinomas and associated immune stroma. *Gut* 2017; **66**: 794-801 [PMID: 26801886 DOI: 10.1136/gutjnl-2015-310839]

59   **Zhou S**, Shen Z, Wang Y, Ma H, Xu S, Qin J, Chen L, Tao H, Zhen Z, Chen G, Zhang Z, Li R, Xiao H, Zhong C, Yang Y, Liang C. CCR7 expression and intratumoral FOXP3^{+} regulatory T cells are correlated with overall survival and lymph node metastasis in gastric cancer. *PLoS One* 2013; **8**: e74430 [PMID: 24040244 DOI: 10.1371/journal.pone.0074430]

60   **Wang B**, Xu D, Yu X, Ding T, Rao H, Zhan Y, Zheng L, Li L. Association of intra-tumoral infiltrating macrophages and regulatory T cells is an independent prognostic factor in gastric cancer after radical resection. *Ann Surg Oncol* 2011; **18**: 2585-2593 [PMID: 21347781 DOI: 10.1245/s10434-011-1609-3]

61   **Perrone G**, Ruffini PA, Catalano V, Spino C, Santini D, Muretto P, Spoto C, Zingaretti C, Sisti V, Alessandroni P, Giordani P, Cicetti A, D'Emidio S, Morini S, Ruzzo A, Magnani M, Tonini G, Rabitti C, Graziano F. Intratumoural FOXP3-positive regulatory T cells

are associated with adverse prognosis in radically resected gastric cancer. *Eur J Cancer* 2008; **44**: 1875-1882 [PMID: 18617393 DOI: 10.1016/j.ejca.2008.05.017]

62   **Kim KJ**, Wen XY, Yang HK, Kim WH, Kang GH. Prognostic Implication of M2 Macrophages Are Determined by the Proportional Balance of Tumor Associated Macrophages and Tumor Infiltrating Lymphocytes in Microsatellite-Unstable Gastric Carcinoma. *PLoS One* 2015; **10**: e0144192 [PMID: 26714314 DOI: 10.1371/journal.pone.0144192]

63   **Hu M**, Li K, Maskey N, Xu Z, Peng C, Wang B, Li Y, Yang G. Decreased intratumoral Foxp3 Tregs and increased dendritic cell density by neoadjuvant chemotherapy associated with favorable prognosis in advanced gastric cancer. *Int J Clin Exp Pathol* 2014; **7**: 4685-4694 [PMID: 25197340]

64   **Mizukami Y**, Kono K, Kawaguchi Y, Akaike H, Kamimura K, Sugai H, Fujii H. Localisation pattern of Foxp3^{+} regulatory T cells is associated with clinical behaviour in gastric cancer. *Br J Cancer* 2008; **98**: 148-153 [PMID: 18087278 DOI: 10.1038/sj.bjc.6604149]

65   **Shen Z**, Zhou S, Wang Y, Li RL, Zhong C, Liang C, Sun Y. Higher intratumoral infiltrated Foxp3^{+} Treg numbers and Foxp3^{+}/CD8^{+} ratio are associated with adverse prognosis in resectable gastric cancer. *J Cancer Res Clin Oncol* 2010; **136**: 1585-1595 [PMID: 20221835 DOI: 10.1007/s00432-010-0816-9]

66   **Liu T**, Peng L, Yu P, Zhao Y, Shi Y, Mao X, Chen W, Cheng P, Wang T, Chen N, Zhang J, Liu X, Li N, Guo G, Tong W, Zhuang Y, Zou Q. Increased circulating Th22 and Th17 cells are associated with tumor progression and patient survival in human gastric cancer. *J Clin Immunol* 2012; **32**: 1332-1339 [PMID: 22760549 DOI: 10.1007/s10875-012-9718-8]

67   **Ubukata H**, Motohashi G, Tabuchi T, Nagata H, Konishi S, Tabuchi T. Evaluations of interferon-γ/interleukin-4 ratio and neutrophil/lymphocyte ratio as prognostic indicators in gastric cancer patients. *J Surg Oncol* 2010; **102**: 742-747 [PMID: 20872813 DOI: 10.1002/jso.21725]

68   **Lee HE**, Chae SW, Lee YJ, Kim MA, Lee HS, Lee BL, Kim WH. Prognostic implications of type and density of tumour-infiltrating lymphocytes in gastric cancer. *Br J Cancer* 2008; **99**: 1704-1711 [PMID: 18941457 DOI: 10.1038/sj.bjc.6604738]

69   **Ishigami S**, Natsugoe S, Tokuda K, Nakajo A, Xiangming C, Iwashige H, Aridome K, Hokita S, Aikou T. Clinical impact of intratumoral natural killer cell and dendritic cell infiltration in gastric cancer. *Cancer Lett* 2000; **159**: 103-108 [PMID: 10974412 DOI: 10.1016/S0304-3835(00)00542-5]

70   **Mimura K**, Kamiya T, Shiraishi K, Kua LF, Shabbir A, So J, Yong WP, Suzuki Y, Yoshimoto Y, Nakano T, Fujii H, Campana D, Kono K. Therapeutic potential of highly cytotoxic natural killer cells for gastric cancer. *Int J Cancer* 2014; **135**: 1390-1398 [PMID: 24615495 DOI: 10.1002/ijc.28780]

71   **Wakatsuki K**, Sho M, Yamato I, Takayama T, Matsumoto S, Tanaka T, Migita K, Ito M, Hotta K, Nakajima Y. Clinical impact of tumor-infiltrating CD45RO^{+} memory T cells on human gastric cancer. *Oncol Rep* 2013; **29**: 1756-1762 [PMID: 23440298 DOI: 10.3892/or.2013.2302]

72   **Chen LJ**, Zheng X, Shen YP, Zhu YB, Li Q, Chen J, Xia R, Zhou SM, Wu CP, Zhang XG, Lu BF, Jiang JT. Higher numbers of T-bet(+) intratumoral lymphoid cells correlate with better survival in gastric cancer. *Cancer Immunol Immunother* 2013; **62**: 553-561 [PMID: 23090288 DOI: 10.1007/s00262-012-1358-6]

73   **Hennequin A**, Derangère V, Boidot R, Apetoh L, Vincent J, Orry D, Fraisse J, Causeret S, Martin F, Arnould L, Beltjens F, Ghiringhelli F, Ladoire S. Tumor infiltration by Tbet^{+} effector T cells and CD20^{+} B cells is associated with survival in gastric cancer patients. *Oncoimmunology* 2015; **5**: e1054598 [PMID: 27057426 DOI: 10.1080/2162402X.2015.1054598]

74   **Arigami T**, Uenosono Y, Ishigami S, Matsushita D, Hirahara T, Yanagita S, Okumura H, Uchikado Y, Nakajo A, Kijima Y, Natsugoe S. Decreased density of CD3^{+} tumor-infiltrating lymphocytes during gastric cancer progression. *J Gastroenterol Hepatol* 2014; **29**: 1435-1441 [PMID: 25587614 DOI: 10.1111/jgh.12551]

75   **Michie CA**, McLean A, Alcock C, Beverley PC. Lifespan of human

76   **Fossiez F**, Djossou O, Chomarat P, Flores-Romo L, Ait-Yahia S, Maat C, Pin JJ, Garrone P, Garcia E, Saeland S, Blanchard D, Gaillard C, Das Mahapatra B, Rouvier E, Golstein P, Banchereau J, Lebecque S. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J Exp Med* 1996; **183**: 2593-2603 [PMID: 8676080 DOI: 10.1084/jem.183.6.2593]

77   **Benchetrit F**, Ciree A, Vives V, Warnier G, Gey A, Sautès-Fridman C, Fossiez F, Haicheur N, Fridman WH, Tartour E. Interleukin-17 inhibits tumor cell growth by means of a T-cell-dependent mechanism. *Blood* 2002; **99**: 2114-2121 [PMID: 11877287 DOI: 10.1182/blood.V99.6.2114]

78   **Antonysamy MA**, Fanslow WC, Fu F, Li W, Qian S, Troutt AB, Thomson AW. Evidence for a role of IL-17 in organ allograft rejection: IL-17 promotes the functional differentiation of dendritic cell progenitors. *J Immunol* 1999; **162**: 577-584 [PMID: 9886435]

79   **Yuan L**, Xu B, Yuan P, Zhou J, Qin P, Han L, Chen G, Wang Z, Run Z, Zhao P, Gao Q. Tumor-infiltrating CD4^{+} T cells in patients with gastric cancer. *Cancer Cell Int* 2017; **17**: 114 [PMID: 29213216 DOI: 10.1186/s12935-017-0489-4]

80   **Choi HS**, Ha SY, Kim HM, Ahn SM, Kang MS, Kim KM, Choi MG, Lee JH, Sohn TS, Bae JM, Kim S, Kang ES. The prognostic effects of tumor infiltrating regulatory T cells and myeloid derived suppressor cells assessed by multicolor flow cytometry in gastric cancer patients. *Oncotarget* 2016; **7**: 7940-7951 [PMID: 26799288 DOI: 10.18632/oncotarget.6958]

81   **Raziorrouh B**, Ulsenheimer A, Schraut W, Heeg M, Kurktschiev P, Zachoval R, Jung MC, Thimme R, Neumann-Haefelin C, Horster S, Wächtler M, Spannagl M, Haas J, Diepolder HM, Grüner NH. Inhibitory molecules that regulate expansion and restoration of HCV-specific CD4^{+} T cells in patients with chronic infection. *Gastroenterology* 2011; **141**: 1422-1431, 1431.e1-1431.e6 [PMID: 21763239 DOI: 10.1053/j.gastro.2011.07.004]

82   **Xu B**, Yuan L, Gao Q, Yuan P, Zhao P, Yuan H, Fan H, Li T, Qin P, Han L, Fang W, Suo Z. Circulating and tumor-infiltrating Tim-3 in patients with colorectal cancer. *Oncotarget* 2015; **6**: 20592-20603 [PMID: 26008981 DOI: 10.18632/oncotarget.4112]

83   **Figdor CG**, de Vries IJ, Lesterhuis WJ, Melief CJ. Dendritic cell immunotherapy: mapping the way. *Nat Med* 2004; **10**: 475-480 [PMID: 15122249 DOI: 10.1038/nm1039]

84   **Tsujitani S**, Kakeji Y, Orita H, Watanabe A, Kohnoe S, Baba H, Anai H, Maehara Y, Sugimachi K. Postoperative adjuvant immunochemotherapy and infiltration of dendritic cells for patients with advanced gastric cancer. *Anticancer Res* 1992; **12**: 645-648 [PMID: 1622120]

85   **Teng MW**, Ngiow SF, Ribas A, Smyth MJ. Classifying Cancers Based on T-cell Infiltration and PD-L1. *Cancer Res* 2015; **75**: 2139-2145 [PMID: 25977340 DOI: 10.1158/0008-5472.CAN-15-0255]

86   **Tumeh PC**, Harview CL, Yearley JH, Shintaku IP, Taylor EJ, Robert L, Chmielowski B, Spasic M, Henry G, Ciobanu V, West AN, Carmona M, Kivork C, Seja E, Cherry G, Gutierrez AJ, Grogan TR, Mateus C, Tomasic G, Glaspy JA, Emerson RO, Robins H, Pierce RH, Elashoff DA, Robert C, Ribas A. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* 2014; **515**: 568-571 [PMID: 25428505 DOI: 10.1038/nature13954]

87   **Ma J**, Li J, Hao Y, Nie Y, Li Z, Qian M, Liang Q, Yu J, Zeng M, Wu K. Differentiated tumor immune microenvironment of Epstein-Barr virus-associated and negative gastric cancer: implication in prognosis and immunotherapy. *Oncotarget* 2017; **8**: 67094-67103 [PMID: 28978018 DOI: 10.18632/oncotarget.17945]

88   **Grogg KL**, Lohse CM, Pankratz VS, Halling KC, Smyrk TC. Lymphocyte-rich gastric cancer: associations with Epstein-Barr virus, microsatellite instability, histology, and survival. *Mod Pathol* 2003; **16**: 641-651 [PMID: 12861059 DOI: 10.1097/01.MP.0000076980.73826.C0]

89   **Ghiringhelli F**, Ménard C, Terme M, Flament C, Taieb J, Chaput N, Puig PE, Novault S, Escudier B, Vivier E, Lecesne A, Robert C, Blay JY, Bernard J, Caillat-Zucman S, Freitas A, Tursz T, Wagner-Ballon O, Capron C, Vainchencker W, Martin F, Zitvogel L.

lymphocyte subsets defined by CD45 isoforms. *Nature* 1992; **360**: 264-265 [PMID: 1436108 DOI: 10.1038/360264a0]

CD4⁺CD25⁺ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. *J Exp Med* 2005; **202**: 1075-1085 [PMID: 16230475 DOI: 10.1084/jem.20051511]

90   **Wang L**, Chang EW, Wong SC, Ong SM, Chong DQ, Ling KL. Increased myeloid-derived suppressor cells in gastric cancer correlate with cancer stage and plasma S100A8/A9 proinflammatory proteins. *J Immunol* 2013; **190**: 794-804 [PMID: 23248262 DOI: 10.4049/jimmunol.1202088]

91   **Almand B**, Clark JI, Nikitina E, van Beynen J, English NR, Knight SC, Carbone DP, Gabrilovich DI. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol* 2001; **166**: 678-689 [PMID: 11123353 DOI: 10.4049/jimmunol.166.1.678]

92   **Shoji H**, Tada K, Kitano S, Nishimura T, Shimada Y, Nagashima K, Aoki K, Hiraoka N, Honma Y, Iwasa S, Takashima A, Kato K, Boku N, Honda K, Yamada T, Heike Y, Hamaguchi T. The peripheral immune status of granulocytic myeloid-derived suppressor cells correlates the survival in advanced gastric cancer patients receiving cisplatin-based chemotherapy. *Oncotarget* 2017; **8**: 95083-95094 [PMID: 29221113 DOI: 10.18632/oncotarget.18297]

93   **Asfaha S**, Dubeykovskiy AN, Tomita H, Yang X, Stokes S, Shibata W, Friedman RA, Ariyama H, Dubeykovskaya ZA, Muthupalani S, Ericksen R, Frucht H, Fox JG, Wang TC. Mice that express human interleukin-8 have increased mobilization of immature myeloid cells, which exacerbates inflammation and accelerates colon carcinogenesis. *Gastroenterology* 2013; **144**: 155-166 [PMID: 23041326 DOI: 10.1053/j.gastro.2012.09.057]

94   **Chi N**, Tan Z, Ma K, Bao L, Yun Z. Increased circulating myeloid-derived suppressor cells correlate with cancer stages, interleukin-8 and -6 in prostate cancer. *Int J Clin Exp Med* 2014; **7**: 3181-3192 [PMID: 25419348]

95   **Coussens LM**, Werb Z. Inflammation and cancer. *Nature* 2002; **420**: 860-867 [PMID: 12490959 DOI: 10.1038/nature01322]

96   **Grivennikov SI**, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* 2010; **140**: 883-899 [PMID: 20303878 DOI: 10.1016/j.cell.2010.01.025]

97   **McMillan DC**. Systemic inflammation, nutritional status and survival in patients with cancer. *Curr Opin Clin Nutr Metab Care* 2009; **12**: 223-226 [PMID: 19318937 DOI: 10.1097/MCO.0b013e32832a7902]

98   **Zahorec R**. Ratio of neutrophil to lymphocyte counts--rapid and simple parameter of systemic inflammation and stress in critically ill. *Bratisl Lek Listy* 2001; **102**: 5-14 [PMID: 11723675]

99   **Shibata M**, Nezu T, Kanou H, Abe H, Takekawa M, Fukuzawa M. Decreased production of interleukin-12 and type 2 immune responses are marked in cachectic patients with colorectal and gastric cancer. *J Clin Gastroenterol* 2002; **34**: 416-420 [PMID: 11907352 DOI: 10.1097/00004836-200204000-00006]

100   **Ohki S**, Shibata M, Gonda K, Machida T, Shimura T, Nakamura I, Ohtake T, Koyama Y, Suzuki S, Ohto H, Takenoshita S. Circulating myeloid-derived suppressor cells are increased and correlate to immune suppression, inflammation and hypoproteinemia in patients with cancer. *Oncol Rep* 2012; **28**: 453-458 [PMID: 22614133 DOI: 10.3892/or.2012.1812]

101   **Gonda K**, Shibata M, Sato Y, Washio M, Takeshita H, Shigeta H, Ogura M, Oka S, Sakuramoto S. Elevated neutrophil-to-lymphocyte ratio is associated with nutritional impairment, immune suppression, resistance to S-1 plus cisplatin, and poor prognosis in patients with stage Ⅳ gastric cancer. *Mol Clin Oncol* 2017; **7**: 1073-1078 [PMID: 29285377 DOI: 10.3892/mco.2017.1438]

102   **McCarthy EF**. The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas. *Iowa Orthop J* 2006; **26**: 154-158 [PMID: 16789469]

103   **Niccolai E**, Taddei A, Prisco D, Amedei A. Gastric cancer and the epoch of immunotherapy approaches. *World J Gastroenterol* 2015; **21**: 5778-5793 [PMID: 26019442 DOI: 10.3748/wjg.v21.i19.5778]

104   **Bonotto M**, Garattini SK, Basile D, Ongaro E, Fanotto V, Cattaneo M, Cortiula F, Iacono D, Cardellino GG, Pella N, Fasola G, Antonuzzo L, Silvestris N, Aprile G. Immunotherapy for gastric

cancers: emerging role and future perspectives. *Expert Rev Clin Pharmacol* 2017; **10**: 609-619 [PMID: 28349740 DOI: 10.1080/17512433.2017.1313113]

105   **Chen L**, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol* 2013; **13**: 227-242 [PMID: 23470321 DOI: 10.1038/nri3405]

106   **Hanna MG Jr**, Hoover HC Jr, Vermorken JB, Harris JE, Pinedo HM. Adjuvant active specific immunotherapy of stage Ⅱ and stage Ⅲ colon cancer with an autologous tumor cell vaccine: first randomized phase Ⅲ trials show promise. *Vaccine* 2001; **19**: 2576-2582 [PMID: 11257395 DOI: 10.1016/S0264-410X(00)00485-0]

107   **Derouazi M**, Di Berardino-Besson W, Belnoue E, Hoepner S, Walther R, Benkhoucha M, Teta P, Dufour Y, Yacoub Maroun C, Salazar AM, Martinvalet D, Dietrich PY, Walker PR. Novel Cell-Penetrating Peptide-Based Vaccine Induces Robust CD4⁺ and CD8⁺ T Cell-Mediated Antitumor Immunity. *Cancer Res* 2015; **75**: 3020-3031 [PMID: 26116496 DOI: 10.1158/0008-5472.CAN-14-3017]

108   **Rosenberg SA**, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, Citrin DE, Restifo NP, Robbins PF, Wunderlich JR, Morton KE, Laurencot CM, Steinberg SM, White DE, Dudley ME. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res* 2011; **17**: 4550-4557 [PMID: 21498393 DOI: 10.1158/1078-0432.CCR-11-0116]

109   **Nie Y**, Wu K, Yang J, Tian F, Li L, Chen B, Fan D. Induction of T lymphocytes specific to human gastric cancer using HLA-A matched allogeneic gastric tumor cells. *J Immunother* 2003; **26**: 403-411 [PMID: 12973029]

110   **Kawamoto M**, Tanaka F, Mimori K, Inoue H, Kamohara Y, Mori M. Identification of HLA-A*0201/-A*2402-restricted CTL epitope-peptides derived from a novel cancer/testis antigen, MCAK, and induction of a specific antitumor immune response. *Oncol Rep* 2011; **25**: 469-476 [PMID: 21165574 DOI: 10.3892/or.2010.1101]

111   **Kono K**, Ichihara F, Iizuka H, Sekikawa T, Matsumoto Y. Differences in the recognition of tumor-specific CD8⁺ T cells derived from solid tumor, metastatic lymph nodes and ascites in patients with gastric cancer. *Int J Cancer* 1997; **71**: 978-981 [PMID: 9185700]

112   **Fujie T**, Tanaka F, Tahara K, Li J, Tanaka S, Mori M, Ueo H, Takesako K, Akiyoshi T. Generation of specific antitumor reactivity by the stimulation of spleen cells from gastric cancer patients with MAGE-3 synthetic peptide. *Cancer Immunol Immunother* 1999; **48**: 189-194 [PMID: 10431688]

113   **Sun S**, Li XM, Li XD, Yang WS. Studies on inducing apoptosis effects and mechanism of CIK cells for MGC-803 gastric cancer cell lines. *Cancer Biother Radiopharm* 2005; **20**: 173-180 [PMID: 15869451 DOI: 10.1089/cbr.2005.20.173]

114   **Kim YJ**, Lim J, Kang JS, Kim HM, Lee HK, Ryu HS, Kim JY, Hong JT, Kim Y, Han SB. Adoptive immunotherapy of human gastric cancer with ex vivo expanded T cells. *Arch Pharm Res* 2010; **33**: 1789-1795 [PMID: 21116782 DOI: 10.1007/s12272-010-1111-7]

115   **Bourquin C**, von der Borch P, Zoglmeier C, Anz D, Sandholzer N, Suhartha N, Wurzenberger C, Denzel A, Kammerer R, Zimmermann W, Endres S. Efficient eradication of subcutaneous but not of autochthonous gastric tumors by adoptive T cell transfer in an SV40 T antigen mouse model. *J Immunol* 2010; **185**: 2580-2588 [PMID: 20644173 DOI: 10.4049/jimmunol.0903231]

116   **Gebremeskel S**, Johnston B. Concepts and mechanisms underlying chemotherapy induced immunogenic cell death: impact on clinical studies and considerations for combined therapies. *Oncotarget* 2015; **6**: 41600-41619 [PMID: 26486085 DOI: 10.18632/oncotarget.6113]

117   **Zhao Q**, Zhang H, Li Y, Liu J, Hu X, Fan L. Anti-tumor effects of CIK combined with oxaliplatin in human oxaliplatin-resistant gastric cancer cells *in vivo* and *in vitro*. *J Exp Clin Cancer Res* 2010; **29**: 118 [PMID: 20799994 DOI: 10.1186/1756-9966-29-118]

118   **Dudley ME**, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, Topalian SL, Sherry R, Restifo NP, Hubicki AM, Robinson MR, Raffeld M, Duray P, Seipp CA, Rogers-Freezer L, Morton KE, Mavroukakis SA, White DE, Rosenberg SA. Cancer regression and autoimmunity in patients after clonal repopulation

with antitumor lymphocytes. *Science* 2002; **298**: 850-854 [PMID: 12242449 DOI: 10.1126/science.1076514]

119 **Kobold S**, Steffen J, Chaloupka M, Grassmann S, Henkel J, Castoldi R, Zeng Y, Chmielewski M, Schmollinger JC, Schnurr M, Rothenfußer S, Schendel DJ, Abken H, Sustmann C, Niederfellner G, Klein C, Bourquin C, Endres S. Selective bispecific T cell recruiting antibody and antitumor activity of adoptive T cell transfer. *J Natl Cancer Inst* 2014; **107**: 364 [PMID: 25424197 DOI: 10.1093/jnci/dju364]

120 **Du X**, Jin R, Ning N, Li L, Wang Q, Liang W, Liu J, Xu Y. In vivo distribution and antitumor effect of infused immune cells in a gastric cancer model. *Oncol Rep* 2012; **28**: 1743-1749 [PMID: 22948809 DOI: 10.3892/or.2012.2013]

121 **Kevin T**, Mark H, Craig L, inventors; Alan J, assignee; Cytotoxic T Lymphocyte-stimulation peptides for prevention, treatment, and diagnosis of melanoma. International patent WO2001032193. 2001 May 10

122 **Bhatia S**, Tykodi SS, Thompson JA. Treatment of metastatic melanoma: an overview. *Oncology (Williston Park)* 2009; **23**: 488-496 [PMID: 19544689]

123 **Yee C**, Thompson JA, Byrd D, Riddell SR, Roche P, Celis E, Greenberg PD. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. *Proc Natl Acad Sci U S A* 2002; **99**: 16168-16173 [PMID: 12427970 DOI: 10.1073/pnas.242600099]

124 **Rosenberg SA**, Lotze MT, Muul LM, Chang AE, Avis FP, Leitman S, Linehan WM, Robertson CN, Lee RE, Rubin JT. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N Engl J Med* 1987; **316**: 889-897 [PMID: 3493432]

125 **Zhang GQ**, Zhao H, Wu JY, Li JY, Yan X, Wang G, Wu LL, Zhang XG, Shao Y, Wang Y, Jiao SC. Prolonged overall survival in gastric cancer patients after adoptive immunotherapy. *World J Gastroenterol* 2015; **21**: 2777-2785 [PMID: 25759549 DOI: 10.3748/wjg.v21.i9.2777]

126 **Zhao H**, Fan Y, Li H, Yu J, Liu L, Cao S, Ren B, Yan F, Ren X. Immunotherapy with cytokine-induced killer cells as an adjuvant treatment for advanced gastric carcinoma: a retrospective study of 165 patients. *Cancer Biother Radiopharm* 2013; **28**: 303-309 [PMID: 23506427 DOI: 10.1089/cbr.2012.1306]

127 **Chen Y**, Guo ZQ, Shi CM, Zhou ZF, Ye YB, Chen Q. Efficacy of adjuvant chemotherapy combined with immunotherapy with cytokine-induced killer cells for gastric cancer after d2 gastrectomy. *Int J Clin Exp Med* 2015; **8**: 7728-7736 [PMID: 26221323]

128 **Shi L**, Zhou Q, Wu J, Ji M, Li G, Jiang J, Wu C. Efficacy of adjuvant immunotherapy with cytokine-induced killer cells in patients with locally advanced gastric cancer. *Cancer Immunol Immunother* 2012; **61**: 2251-2259 [PMID: 22674056 DOI: 10.1007/s00262-012-1289-2]

129 **Jiang J**, Xu N, Wu C, Deng H, Lu M, Li M, Xu B, Wu J, Wang R, Xu J, Nilsson-Ehle P. Treatment of advanced gastric cancer by chemotherapy combined with autologous cytokine-induced killer cells. *Anticancer Res* 2006; **26**: 2237-2242 [PMID: 16821594]

130 **Jiang JT**, Shen YP, Wu CP, Zhu YB, Wei WX, Chen LJ, Zheng X, Sun J, Lu BF, Zhang XG. Increasing the frequency of CIK cells adoptive immunotherapy may decrease risk of death in gastric cancer patients. *World J Gastroenterol* 2010; **16**: 6155-6162 [PMID: 21182234 DOI: 10.3748/wjg.v16.i48.6155]

131 **Jäkel CE**, Vogt A, Gonzalez-Carmona MA, Schmidt-Wolf IG. Clinical studies applying cytokine-induced killer cells for the treatment of gastrointestinal tumors. *J Immunol Res* 2014; **2014**: 897214 [PMID: 24741629 DOI: 10.1155/2014/897214]

132 **Wada I**, Matsushita H, Noji S, Mori K, Yamashita H, Nomura S, Shimizu N, Seto Y, Kakimi K. Intraperitoneal injection of *in vitro* expanded Vγ9Vδ2 T cells together with zoledronate for the treatment of malignant ascites due to gastric cancer. *Cancer Med* 2014; **3**: 362-375 [PMID: 24515916 DOI: 10.1002/cam4.196]

133 **Cellular Biomedicine Group Ltd**. The Study of Surgery, Chemotherapy and Autologous T Cells-Based Immunotherapy for Advanced Gastric Cancer. [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT02704299 ClinicalTrials.gov Identifier: NCT02704299

134 **Shenzhen Hornetcorn Bio-technology Company, LTD**. "Study of Autologous Tumor Lysate-pulsed D-CIK Combined With Chemotherapy for Gastric Cancer". [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT02215837 ClinicalTrials.gov Identifier: NCT02215837

135 **First Affiliated Hospital of Chengdu Medical College**. A Clinical Research of CAR T Cells Targeting EpCAMPositive Cancer (CARTEPC). [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT03013712 ClinicalTrials.gov Identifier: NCT03013712

136 **PersonGen BioTherapeutics (Suzhou) Co., Ltd**. CAR-T Cell Immunotherapy in MUC1 Positive Solid Tumor. [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT02617134 ClinicalTrials.gov Identifier: NCT02617134

137 **Roger Williams Medical Center**. CAR-T Hepatic Artery Infusions for CEA-Expressing Liver Metastases (HITM-SURE). [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT02850536 ClinicalTrials.gov Identifier: NCT02850536

138 **University SMM,** Immunotherapy Using Pluripotent Killer-Human Epidermal Growth Factor Receptor-2 (PIK-HER2) Cells for the Treatment of Advanced Gastric Cancer With Liver Metastasis. [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT02632201 ClinicalTrials.gov Identifier: NCT02632201

139 **Miao Y**. T Cell Mediated Adaptive therapy for Her2-positive Neoplasms of Digestive System, in ClinicalTrials.gov [Internet], Bethesda (MD): National Library of Medicine (US), 2000 Available from: URL: http://clinicaltrials.gov/show/NCT02662348

140 **Capital Medical University.** Study of S-1 Plus DC-CIK for Patients With Advanced Gastric Cancer. [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT01783951 ClinicalTrials.gov Identifier: NCT01783951

141 **Dudley ME**, Wunderlich JR, Shelton TE, Even J, Rosenberg SA. Generation of tumor-infiltrating lymphocyte cultures for use in adoptive transfer therapy for melanoma patients. *J Immunother* 2003; **26**: 332-342 [PMID: 12843795 DOI: 10.1097/00002371-200307000-00005]

142 **Alexander RB**, Rosenberg SA. Long-term survival of adoptively transferred tumor-infiltrating lymphocytes in mice. *J Immunol* 1990; **145**: 1615-1620 [PMID: 1974569]

143 **Kono K**, Takahashi A, Ichihara F, Amemiya H, Iizuka H, Fujii H, Sekikawa T, Matsumoto Y. Prognostic significance of adoptive immunotherapy with tumor-associated lymphocytes in patients with advanced gastric cancer: a randomized trial. *Clin Cancer Res* 2002; **8**: 1767-1771 [PMID: 12060615]

144 **Rosenberg SA**, Yannelli JR, Yang JC, Topalian SL, Schwartzentruber DJ, Weber JS, Parkinson DR, Seipp CA, Einhorn JH, White DE. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J Natl Cancer Inst* 1994; **86**: 1159-1166 [PMID: 8028037 DOI: 10.1093/jnci/86.15.1159]

145 **Amedei A**, Munari F, Bella CD, Niccolai E, Benagiano M, Bencini L, Cianchi F, Farsi M, Emmi G, Zanotti G, de Bernard M, Kundu M, D'Elios MM. Helicobacter pylori secreted peptidyl prolyl cis, trans-isomerase drives Th17 inflammation in gastric adenocarcinoma. *Intern Emerg Med* 2014; **9**: 303-309 [PMID: 23054412 DOI: 10.1007/s11739-012-0867-9]

146 **Yamaue H**, Tanimura H, Tsunoda T, Iwahashi M, Tani M, Inoue M, Tamai M. [Clinical application of adoptive immunotherapy

by cytotoxic T lymphocytes induced from tumor-infiltrating lymphocytes]. *Nihon Gan Chiryo Gakkai Shi* 1990; **25**: 978-989 [PMID: 2391445]

147 **Voskens CJ**, Watanabe R, Rollins S, Campana D, Hasumi K, Mann DL. Ex-vivo expanded human NK cells express activating receptors that mediate cytotoxicity of allogeneic and autologous cancer cell lines by direct recognition and antibody directed cellular cytotoxicity. *J Exp Clin Cancer Res* 2010; **29**: 134 [PMID: 20937115 DOI: 10.11 86/1756-9966-29-134]

148 **Langers I**, Renoux VM, Thiry M, Delvenne P, Jacobs N. Natural killer cells: role in local tumor growth and metastasis. *Biologics* 2012; **6**: 73-82 [PMID: 22532775 DOI: 10.2147/BTT.S23976]

149 **Kim S**, Iizuka K, Aguila HL, Weissman IL, Yokoyama WM. In vivo natural killer cell activities revealed by natural killer cell-deficient mice. *Proc Natl Acad Sci USA* 2000; **97**: 2731-2736 [PMID: 10694580 DOI: 10.1073/pnas.050588297]

150 **Coca S**, Perez-Piqueras J, Martinez D, Colmenarejo A, Saez MA, Vallejo C, Martos JA, Moreno M. The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. *Cancer* 1997; **79**: 2320-2328 [PMID: 9191519 DOI: 10.1002/(SICI) 1097-0142(19970615)79:12<2320::AID-CNCR5>3.0.CO;2-P]

151 **Rosso D**, Rigueiro MP, Kassab P, Ilias EJ, Castro OA, Novo NF, Lourenço LG. [Correlation of natural killer cells with the prognosis of gastric adenocarcinoma]. *Arq Bras Cir Dig* 2012; **25**: 114-117 [PMID: 23381755 DOI: 10.1590/S0102-67202012000200011]

152 **Saito H**, Takaya S, Osaki T, Ikeguchi M. Increased apoptosis and elevated Fas expression in circulating natural killer cells in gastric cancer patients. *Gastric Cancer* 2013; **16**: 473-479 [PMID: 23179366 DOI: 10.1007/s10120-012-0210-1]

153 **Cui J**, Li L, Wang C, Jin H, Yao C, Wang Y, Li D, Tian H, Niu C, Wang G, Han W, Xu J, Chen J, Li W. Combined cellular immunotherapy and chemotherapy improves clinical outcome in patients with gastric carcinoma. *Cytotherapy* 2015; **17**: 979-988 [PMID: 25890480 DOI: 10.1016/j.jcyt.2015.03.605]

154 **National University Hospital, Singapore**. NK cell infusions with trastuzumab for patients with HER2$^+$ breast and gastric cancer". [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http:// clinicaltrials.gov/show/NCT02030561 ClinicalTrials.gov Identifier: NCT02030561

155 **Wu XT**, Liu JQ, Lu XT, Chen FX, Zhou ZH, Wang T, Zhu SP, Fei SJ. The enhanced effect of lupeol on the destruction of gastric cancer cells by NK cells. *Int Immunopharmacol* 2013; **16**: 332-340 [PMID: 23639256 DOI: 10.1016/j.intimp.2013.04.017]

156 **Kono K**, Rongcun Y, Charo J, Ichihara F, Celis E, Sette A, Appella E, Sekikawa T, Matsumoto Y, Kiessling R. Identification of HER2/ neu-derived peptide epitopes recognized by gastric cancer-specific cytotoxic T lymphocytes. *Int J Cancer* 1998; **78**: 202-208 [PMID: 9754653 DOI: 10.1002/(SICI)1097-0215(19981005)78:2<202:: AID-IJC14>3.0.CO;2-C]

157 **Tanaka F**, Fujie T, Tahara K, Mori M, Takesako K, Sette A, Celis E, Akiyoshi T. Induction of antitumor cytotoxic T lymphocytes with a MAGE-3-encoded synthetic peptide presented by human leukocytes antigen-A24. *Cancer Res* 1997; **57**: 4465-4468 [PMID: 9377553]

158 **Higashihara Y**, Kato J, Nagahara A, Izumi K, Konishi M, Kodani T, Serizawa N, Osada T, Watanabe S. Phase I clinical trial of peptide vaccination with URLC10 and VEGFR1 epitope peptides in patients with advanced gastric cancer. *Int J Oncol* 2014; **44**: 662-668 [PMID: 24398900 DOI: 10.3892/ijo.2013.2242]

159 **Gang Y**, Zhang X, He Y, Zheng J, Wu K, Ding J, Fan D. Efficient induction of specific cytotoxic T lymphocytes against gastric adenocarcinoma by a survivin peptide. *Biochem Cell Biol* 2012; **90**: 701-708 [PMID: 22992138 DOI: 10.1139/o2012-028]

160 **Zhang K**, Peng Z, Huang X, Qiao Z, Wang X, Wang N, Xi H, Cui J, Gao Y, Huang X, Gao H, Wei B, Chen L. Phase II Trial of Adjuvant Immunotherapy with Autologous Tumor-derived Gp96 Vaccination in Patients with Gastric Cancer. *J Cancer* 2017; **8**: 1826-1832 [PMID: 28819380 DOI: 10.7150/jca.18946]

161 **Celluzzi CM**, Mayordomo JI, Storkus WJ, Lotze MT, Falo LD Jr. Peptide-pulsed dendritic cells induce antigen-specific CTL-mediated protective tumor immunity. *J Exp Med* 1996; **183**: 283-287 [PMID: 8551233 DOI: 10.1084/jem.183.1.283]

162 **Gilboa E**. DC-based cancer vaccines. *J Clin Invest* 2007; **117**: 1195-1203 [PMID: 17476349 DOI: 10.1172/JCI31205]

163 **Yang J**, Li ZH, Zhou JJ, Chen RF, Cheng LZ, Zhou QB, Yang LQ. Preparation and antitumor effects of nanovaccines with MAGE-3 peptides in transplanted gastric cancer in mice. *Chin J Cancer* 2010; **29**: 359-364 [PMID: 20346208 DOI: 10.5732/cjc.009.10541]

164 **Galetto A**, Contarini M, Sapino A, Cassoni P, Consalvo E, Forno S, Pezzi C, Barnaba V, Mussa A, Matera L. Ex vivo host response to gastrointestinal cancer cells presented by autologous dendritic cells. *J Surg Res* 2001; **100**: 32-38 [PMID: 11516202 DOI: 10.1006/ jsre.2001.6158]

165 **Kreiter S**, Selmi A, Diken M, Koslowski M, Britten CM, Huber C, Türeci O, Sahin U. Intranodal vaccination with naked antigen-encoding RNA elicits potent prophylactic and therapeutic antitumoral immunity. *Cancer Res* 2010; **70**: 9031-9040 [PMID: 21045153 DOI: 10.1158/0008-5472.CAN-10-0699]

166 **Fotin-Mleczek M**, Duchardt KM, Lorenz C, Pfeiffer R, Ojkić-Zrna S, Probst J, Kallen KJ. Messenger RNA-based vaccines with dual activity induce balanced TLR-7 dependent adaptive immune responses and provide antitumor activity. *J Immunother* 2011; **34**: 1-15 [PMID: 21150709 DOI: 10.1097/CJI.0b013e3181f7dbe8]

167 **Weide B**, Carralot JP, Reese A, Scheel B, Eigentler TK, Hoerr I, Rammensee HG, Garbe C, Pascolo S. Results of the first phase I/II clinical vaccination trial with direct injection of mRNA. *J Immunother* 2008; **31**: 180-188 [PMID: 18481387 DOI: 10.1097/ CJI.0b013e31815ce501]

168 **Weide B**, Pascolo S, Scheel B, Derhovanessian E, Pflugfelder A, Eigentler TK, Pawelec G, Hoerr I, Rammensee HG, Garbe C. Direct injection of protamine-protected mRNA: results of a phase 1/2 vaccination trial in metastatic melanoma patients. *J Immunother* 2009; **32**: 498-507 [PMID: 19609242 DOI: 10.1097/ CJI.0b013e3181a00068]

169 **Kim TW**, Hung CF, Ling M, Juang J, He L, Hardwick JM, Kumar S, Wu TC. Enhancing DNA vaccine potency by coadministration of DNA encoding antiapoptotic proteins. *J Clin Invest* 2003; **112**: 109-117 [PMID: 12840065 DOI: 10.1172/JCI17293]

170 **Leslie NR**, Batty IH, Maccario H, Davidson L, Downes CP. Understanding PTEN regulation: PIP2, polarity and protein stability. *Oncogene* 2008; **27**: 5464-5476 [PMID: 18794881 DOI: 10.1038/ onc.2008.243]

171 **Kim JH**, Kang TH, Noh KH, Kim SH, Lee YH, Kim KW, Bae HC, Ahn YH, Choi EY, Kim JS, Lee KM, Kim TW. Enhancement of DC vaccine potency by activating the PI3K/AKT pathway with a small interfering RNA targeting PTEN. *Immunol Lett* 2010; **134**: 47-54 [PMID: 20727912 DOI: 10.1016/j.imlet.2010.08.008]

172 **Kim JH**, Kang TH, Noh KH, Bae HC, Ahn YH, Lee YH, Choi EY, Chun KH, Lee SJ, Kim TW. Blocking the immunosuppressive axis with small interfering RNA targeting interleukin (IL)-10 receptor enhances dendritic cell-based vaccine potency. *Clin Exp Immunol* 2011; **165**: 180-189 [PMID: 21592111 DOI: 10.1111/ j.1365-2249.2011.04410.x]

173 **He SB**, Sun K, Wang L, Li DC, Zhang YY. [GM-CSF gene-modified dendritic cell vaccine enhances antitumor immunity in vitro]. *Zhonghua Zhong Liu Za Zhi* 2010; **32**: 410-414 [PMID: 20819479]

174 **Dannull J**, Su Z, Rizzieri D, Yang BK, Coleman D, Yancey D, Zhang A, Dahm P, Chao N, Gilboa E, Vieweg J. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J Clin Invest* 2005; **115**: 3623-3633 [PMID: 16308572 DOI: 10.1172/JCI25947]

175 **Popiela T**, Kulig J, Czupryna A, Szczepanik AM, Zembala M. Efficiency of adjuvant immunochemotherapy following curative resection in patients with locally advanced gastric cancer. *Gastric Cancer* 2004; **7**: 240-245 [PMID: 15616772 DOI: 10.1007/s10120-004-0299-y]

176 **Ajani JA**, Hecht JR, Ho L, Baker J, Oortgiesen M, Eduljee A, Michaeli D. An open-label, multinational, multicenter study of G17DT vaccination combined with cisplatin and 5-fluorouracil in patients with untreated, advanced gastric or gastroesophageal cancer:

the GC4 study. *Cancer* 2006; **106**: 1908-1916 [PMID: 16568451 DOI: 10.1002/cncr.21814]

177 **Masuzawa T**, Fujiwara Y, Okada K, Nakamura A, Takiguchi S, Nakajima K, Miyata H, Yamasaki M, Kurokawa Y, Osawa R, Takeda K, Yoshida K, Tsunoda T, Nakamura Y, Mori M, Doki Y. Phase I/II study of S-1 plus cisplatin combined with peptide vaccines for human vascular endothelial growth factor receptor 1 and 2 in patients with advanced gastric cancer. *Int J Oncol* 2012; **41**: 1297-1304 [PMID: 22842485 DOI: 10.3892/ijo.2012.1573]

178 **Finn OJ**. Cancer immunology. *N Engl J Med* 2008; **358**: 2704-2715 [PMID: 18565863 DOI: 10.1056/NEJMra072739]

179 **Simpson TR**, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, Roddie C, Henry JY, Yagita H, Wolchok JD, Peggs KS, Ravetch JV, Allison JP, Quezada SA. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J Exp Med* 2013; **210**: 1695-1710 [PMID: 23897981 DOI: 10.1084/jem.20130579]

180 **Freeman GJ**, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, Horton HF, Fouser L, Carter L, Ling V, Bowman MR, Carreno BM, Collins M, Wood CR, Honjo T. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 2000; **192**: 1027-1034 [PMID: 11015443 DOI: 10.1084/jem.192.7.1027]

181 **Wang J**, Yuan R, Song W, Sun J, Liu D, Li Z. PD-1, PD-L1 (B7-H1) and Tumor-Site Immune Modulation Therapy: The Historical Perspective. *J Hematol Oncol* 2017; **10**: 34 [PMID: 28122590 DOI: 10.1186/s13045-017-0403-5]

182 **Brahmer JR**, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, Pitot HC, Hamid O, Bhatia S, Martins R, Eaton K, Chen S, Salay TM, Alaparthy S, Grosso JF, Korman AJ, Parker SM, Agrawal S, Goldberg SM, Pardoll DM, Gupta A, Wigginton JM. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med* 2012; **366**: 2455-2465 [PMID: 22658128 DOI: 10.1056/NEJMoa1200694]

183 **Savabkar S**, Azimzadeh P, Chaleshi V, Nazemalhosseini Mojarad E, Aghdaei HA. Programmed death-1 gene polymorphism (PD-1.5 C/T) is associated with gastric cancer. *Gastroenterol Hepatol Bed Bench* 2013; **6**: 178-182 [PMID: 24834269]

184 **Tang W**, Chen Y, Chen S, Sun B, Gu H, Kang M. Programmed death-1 (PD-1) polymorphism is associated with gastric cardia adenocarcinoma. *Int J Clin Exp Med* 2015; **8**: 8086-8093 [PMID: 26221374]

185 **Yan Q**, Chen P, Lu A, Zhao P, Gu A. Association between CTLA-4 60G/A and -1661A/G polymorphisms and the risk of cancers: a meta-analysis. *PLoS One* 2013; **8**: e83710 [PMID: 24376736 DOI: 10.1371/journal.pone.0083710]

186 **Kordi-Tamandani DM**, Davani SK, Baranzehi T, Hemati S. Analysis of promoter methylation, polymorphism and expression profile of cytotoxic T-lymphocyte-associated antigen-4 in patients with gastric cancer. *J Gastrointestin Liver Dis* 2014; **23**: 249-253 [PMID: 25267951 DOI: 10.15403/jgld.2014.1121.233.dmkt]

187 **Saito H**, Kuroda H, Matsunaga T, Osaki T, Ikeguchi M. Increased PD-1 expression on CD4$^+$ and CD8$^+$ T cells is involved in immune evasion in gastric cancer. *J Surg Oncol* 2013; **107**: 517-522 [PMID: 23129549 DOI: 10.1002/jso.23281]

188 **Wu C**, Zhu Y, Jiang J, Zhao J, Zhang XG, Xu N. Immunohistochemical localization of programmed death-1 ligand-1 (PD-L1) in gastric carcinoma and its clinical significance. *Acta Histochem* 2006; **108**: 19-24 [PMID: 16530813 DOI: 10.1016/j.acthis.2006.01.003]

189 **Gu L**, Chen M, Guo D, Zhu H, Zhang W, Pan J, Zhong X, Li X, Qian H, Wang X. PD-L1 and gastric cancer prognosis: A systematic review and meta-analysis. *PLoS One* 2017; **12**: e0182692 [PMID: 28796808 DOI: 10.1371/journal.pone.0182692]

190 **Parsa AT**, Waldron JS, Panner A, Crane CA, Parney IF, Barry JJ, Cachola KE, Murray JC, Tihan T, Jensen MC, Mischel PS, Stokoe D, Pieper RO. Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. *Nat Med* 2007;

191 **Marzec M**, Zhang Q, Goradia A, Raghunath PN, Liu X, Paessler M, Wang HY, Wysocka M, Cheng M, Ruggeri BA, Wasik MA. Oncogenic kinase NPM/ALK induces through STAT3 expression of immunosuppressive protein CD274 (PD-L1, B7-H1). *Proc Natl Acad Sci U S A* 2008; **105**: 20852-20857 [PMID: 19088198 DOI: 10.1073/pnas.0810958105]

192 **Chen L**, Gibbons DL, Goswami S, Cortez MA, Ahn YH, Byers LA, Zhang X, Yi X, Dwyer D, Lin W, Diao L, Wang J, Roybal J, Patel M, Ungewiss C, Peng D, Antonia S, Mediavilla-Varela M, Robertson G, Suraokar M, Welsh JW, Erez B, Wistuba II, Chen L, Peng D, Wang S, Ullrich SE, Heymach JV, Kurie JM, Qin FX. Metastasis is regulated via microRNA-200/ZEB1 axis control of tumour cell PD-L1 expression and intratumoral immunosuppression. *Nat Commun* 2014; **5**: 5241 [PMID: 25348003 DOI: 10.1038/ncomms6241]

193 **Mimura K**, Teh JL, Okayama H, Shiraishi K, Kua LF, Koh V, Smoot DT, Ashktorab H, Oike T, Suzuki Y, Fazreen Z, Asuncion BR, Shabbir A, Yong WP, So J, Soong R, Kono K. PD-L1 expression is mainly regulated by interferon gamma associated with JAK-STAT pathway in gastric cancer. *Cancer Sci* 2018; **109**: 43-53 [PMID: 29034543 DOI: 10.1111/cas.13424]

194 **Böger C**, Behrens HM, Mathiak M, Krüger S, Kalthoff H, Röcken C. PD-L1 is an independent prognostic predictor in gastric cancer of Western patients. *Oncotarget* 2016; **7**: 24269-24283 [PMID: 27009855 DOI: 10.18632/oncotarget.8169]

195 **Kim JW**, Nam KH, Ahn SH, Park DJ, Kim HH, Kim SH, Chang H, Lee JO, Kim YJ, Lee HS, Kim JH, Bang SM, Lee JS, Lee KW. Prognostic implications of immunosuppressive protein expression in tumors as well as immune cell infiltration within the tumor microenvironment in gastric cancer. *Gastric Cancer* 2016; **19**: 42-52 [PMID: 25424150 DOI: 10.1007/s10120-014-0440-5]

196 **Chang H**, Jung WY, Kang Y, Lee H, Kim A, Kim HK, Shin BK, Kim BH. Programmed death-ligand 1 expression in gastric adenocarcinoma is a poor prognostic factor in a high CD8$^+$ tumor infiltrating lymphocytes group. *Oncotarget* 2016; **7**: 80426-80434 [PMID: 27741514 DOI: 10.18632/oncotarget.12603]

197 **Sun J**, Xu K, Wu C, Wang Y, Hu Y, Zhu Y, Chen Y, Shi Q, Yu G, Zhang X. PD-L1 expression analysis in gastric carcinoma tissue and blocking of tumor-associated PD-L1 signaling by two functional monoclonal antibodies. *Tissue Antigens* 2007; **69**: 19-27 [PMID: 17212704 DOI: 10.1111/j.1399-0039.2006.00701.x]

198 **Schlößer HA**, Drebber U, Kloth M, Thelen M, Rothschild SI, Haase S, Garcia-Marquez M, Wennhold K, Berlth F, Urbanski A, Alakus H, Schauss A, Shimabukuro-Vornhagen A, Theurich S, Warnecke-Ebertz U, Stippel DL, Zippelius A, Büttner R, Hallek M, Hölscher AH, Zander T, Mönig SP, von Bergwelt-Baildon M. Immune checkpoints programmed death 1 ligand 1 and cytotoxic T lymphocyte associated molecule 4 in gastric adenocarcinoma. *Oncoimmunology* 2015; **5**: e1100789 [PMID: 27467911 DOI: 10.1080/2162402X.2015.1100789]

199 **Kawazoe A**, Kuwata T, Kuboki Y, Shitara K, Nagatsuma AK, Aizawa M, Yoshino T, Doi T, Ohtsu A, Ochiai A. Clinicopathological features of programmed death ligand 1 expression with tumor-infiltrating lymphocyte, mismatch repair, and Epstein-Barr virus status in a large cohort of gastric cancer patients. *Gastric Cancer* 2017; **20**: 407-415 [PMID: 27629881 DOI: 10.1007/s10120-016-0631-3]

200 **Wu P**, Wu D, Li L, Chai Y, Huang J. PD-L1 and Survival in Solid Tumors: A Meta-Analysis. *PLoS One* 2015; **10**: e0131403 [PMID: 26114883 DOI: 10.1371/journal.pone.0131403]

201 **Xu F**, Feng G, Zhao H, Liu F, Xu L, Wang Q, An G. Clinicopathologic Significance and Prognostic Value of B7 Homolog 1 in Gastric Cancer: A Systematic Review and Meta-Analysis. *Medicine (Baltimore)* 2015; **94**: e1911 [PMID: 26512615 DOI: 10.1097/MD.0000000000001911]

202 **Liu YX**, Wang XS, Wang YF, Hu XC, Yan JQ, Zhang YL, Wang W, Yang RJ, Feng YY, Gao SG, Feng XS. Prognostic significance of PD-L1 expression in patients with gastric cancer in East Asia: a meta-analysis. *Onco Targets Ther* 2016; **9**: 2649-2654 [PMID: 27226727 DOI: 10.2147/OTT.S102616]

203 **Zhang M**, Dong Y, Liu H, Wang Y, Zhao S, Xuan Q, Wang Y, Zhang Q. The clinicopathological and prognostic significance of PD-L1 expression in gastric cancer: a meta-analysis of 10 studies with 1,901 patients. *Sci Rep* 2016; **6**: 37933 [PMID: 27892511 DOI: 10.1038/srep37933]

204 **Fang W**, Chen Y, Sheng J, Zhou T, Zhang Y, Zhan J, Liu L, Huang J, Peng P, Zhang L. Association between PD-L1 Expression on Tumour-Infiltrating Lymphocytes and Overall Survival in Patients with Gastric Cancer. *J Cancer* 2017; **8**: 1579-1585 [PMID: 28775777 DOI: 10.7150/jca.18729]

205 **Gao Y**, Li S, Xu D, Chen S, Cai Y, Jiang W, Zhang X, Sun J, Wang K, Chang B, Wang F, Hong M. Prognostic value of programmed death-1, programmed death-ligand 1, programmed death-ligand 2 expression, and CD8($^+$) T cell density in primary tumors and metastatic lymph nodes from patients with stage T1-4N+M0 gastric adenocarcinoma. *Chin J Cancer* 2017; **36**: 61 [PMID: 28754154 DOI: 10.1186/s40880-017-0226-3]

206 **Ma C**, Patel K, Singhi AD, Ren B, Zhu B, Shaikh F, Sun W. Programmed Death-Ligand 1 Expression Is Common in Gastric Cancer Associated With Epstein-Barr Virus or Microsatellite Instability. *Am J Surg Pathol* 2016; **40**: 1496-1506 [PMID: 27465786 DOI: 10.1097/PAS.0000000000000698]

207 **Budczies J**, Denkert C, Győrffy B, Schirmacher P, Stenzinger A. Chromosome 9p copy number gains involving PD-L1 are associated with a specific proliferation and immune-modulating gene expression program active across major cancer types. *BMC Med Genomics* 2017; **10**: 74 [PMID: 29212506 DOI: 10.1186/s12920-017-0308-8]

208 **Ronan JK.** Immunotherapy for Esophageal and Gastric Cancer. ASCO EDUCATIONAL BOOK | asco.org/edbook. 2017: 292-300

209 **Spranger S**, Gajewski TF. Tumor-intrinsic oncogene pathways mediating immune avoidance. *Oncoimmunology* 2015; **5**: e1086862 [PMID: 27141343 DOI: 10.1080/2162402X.2015.1086862]

210 **Gajewski TF**. The Next Hurdle in Cancer Immunotherapy: Overcoming the Non-T-Cell-Inflamed Tumor Microenvironment. *Semin Oncol* 2015; **42**: 663-671 [PMID: 26320069 DOI: 10.1053/j.seminoncol.2015.05.011]

211 **Tran PN**, Sarkissian S, Chao J, Klempner SJ. PD-1 and PD-L1 as emerging therapeutic targets in gastric cancer: current evidence. *Gastrointest Cancer* 2017; **7**: 1-11 [PMID: 28757801 DOI: 10.2147/GICTT.S113525]

212 **Reck M**, Rodríguez-Abreu D, Robinson AG, Hui R, Csőszi T, Fülöp A, Gottfried M, Peled N, Tafreshi A, Cuffe S, O'Brien M, Rao S, Hotta K, Leiby MA, Lubiniecki GM, Shentu Y, Rangwala R, Brahmer JR; KEYNOTE-024 Investigators. Pembrolizumab versus Chemotherapy for PD-L1-Positive Non-Small-Cell Lung Cancer. *N Engl J Med* 2016; **375**: 1823-1833 [PMID: 27718847 DOI: 10.1056/NEJMoa1606774]

213 **Taube JM**, Klein A, Brahmer JR, Xu H, Pan X, Kim JH, Chen L, Pardoll DM, Topalian SL, Anders RA. Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. *Clin Cancer Res* 2014; **20**: 5064-5074 [PMID: 24714771 DOI: 10.1158/1078-0432.CCR-13-3271]

214 **Jiang Y**, Zhang Q, Hu Y, Li T, Yu J, Zhao L, Ye G, Deng H, Mou T, Cai S, Zhou Z, Liu H, Chen G, Li G, Qi X. ImmunoScore Signature: A Prognostic and Predictive Tool in Gastric Cancer. *Ann Surg* 2018; **267**: 504-513 [PMID: 28002059 DOI: 10.1097/SLA.0000000000002116]

215 **Lin SJ**, Gagnon-Bartsch JA, Tan IB, Earle S, Ruff L, Pettinger K, Ylstra B, van Grieken N, Rha SY, Chung HC, Lee JS, Cheong JH, Noh SH, Aoyama T, Miyagi Y, Tsuburaya A, Yoshikawa T, Ajani JA, Boussioutas A, Yeoh KG, Yong WP, So J, Lee J, Kang WK, Kim S, Kameda Y, Arai T, Zur Hausen A, Speed TP, Grabsch HI, Tan P. Signatures of tumour immunity distinguish Asian and non-Asian gastric adenocarcinomas. *Gut* 2015; **64**: 1721-1731 [PMID: 25385008 DOI: 10.1136/gutjnl-2014-308252]

216 **Ralph C**, Elkord E, Burt DJ, O'Dwyer JF, Austin EB, Stern PL, Hawkins RE, Thistlethwaite FC. Modulation of lymphocyte regulation for cancer therapy: a phase II trial of tremelimumab in advanced gastric and esophageal adenocarcinoma. *Clin Cancer Res* 2010; **16**: 1662-1672 [PMID: 20179239 DOI: 10.1158/1078-0432.CCR-09-2870]

217 **Culver ME**, Gatesman ML, Mancl EE, Lowe DK. Ipilimumab: a novel treatment for metastatic melanoma. *Ann Pharmacother* 2011; **45**: 510-519 [PMID: 21505108 DOI: 10.1345/aph.1P651]

218 **Moehler MH**, Cho JY, Kim YH, Kim JW, Di Bartolomeo M, Ajani JA, Yamaguchi K, Balogh A, Kong-Sanchez MT, Bang YJ. A randomized, open-label, two-arm phase II trial comparing the efficacy of sequential ipilimumab (ipi) versus best supportive care (BSC) following first-line (1L) chemotherapy in patients with unresectable, locally advanced/metastatic (A/M) gastric or gastro-esophageal junction (G/GEJ) cancer. Alexandria: *J Clin Oncol* 2016; **34**: abstr 4011

219 **Janjigian YY**, Ott PA, Calvo E, Kim JW, Ascierto PA, Sharma P, Peltola KJ, Jaeger D,Evans TRJ, De Braud FG, Chau I, Tschaika M, Harbison CT, Cai W, Bendell JC , Le DT.Nivolumab ± ipilimumab in pts with advanced (adv)/metastatic chemotherapy refractory (CTx-R) gastric (G), esophageal (E), or gastroesophageal junction (GEJ) cancer: CheckMate 032 study. *J Clin Oncol* 2017; **35**: abstract 4014

220 **Janjigian YY**, Adenis A, Aucoin JS, Barone C, Boku N, Chau I, Cleary JM, Feeney K, Franke FA, Moehler M, Roca EL, Schenker M, Li M, Ajani JA. Checkmate 649: a randomized, multicenter, open-label, phase 3 study of nivolumab (Nivo) plus ipilimumab (Ipi) versus oxaliplatin plus fluoropyrimidine in patients (Pts) with previously untreated advanced or metastatic gastric (G) or gastroesophageal junction (GEJ) cancer. *J Clin Oncol* 2017; **35**: TPS4132 [DOI: 10.1200/JCO.2017.35.4_suppl.TPS213]

221 **Kang YK**, Satoh T, Ryu MH, Chao Y, Kato K, Chung HC, Chen JS, Muro K, Kang WK, Yoshikawa T, Oh SC, Tamura T, Lee KW, Boku N, Chenet LT. Nivolumab (ONO-4538/BMS-936558) as salvage treatment after second or later-line chemotherapy for advanced gastric or gastro-esophageal junction cancer (AGC): a double-blinded, randomized, phase III trial. *J Clin Oncol* 2017; **35**: abstr 2

222 **Kang YK**, Boku N, Satoh T, Ryu MH, Chao Y, Kato K, Chung HC, Chen JS, Muro K, Kang WK, Yeh KH, Yoshikawa T, Oh SC, Bai LY, Tamura T, Lee KW, Hamamoto Y, Kim JG, Chin K, Oh DY, Minashi K, Cho JY, Tsuda M, Chen LT. Nivolumab in patients with advanced gastric or gastro-oesophageal junction cancer refractory to, or intolerant of, at least two previous chemotherapy regimens (ONO-4538-12, ATTRACTION-2): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet* 2017; **390**: 2461-2471 [PMID: 28993052 DOI: 10.1016/S0140-6736(17)31827-5]

223 **Charalampakis N**, Economopoulou P, Kotsantis I, Tolia M, Schizas D, Liakakos T, Elimova E, Ajani JA, Psyrri A. Medical management of gastric cancer: a 2017 update. *Cancer Med* 2018; **7**: 123-133 [PMID: 29239137 DOI: 10.1002/cam4.1274]

224 **Emens LA**, Middleton G. The interplay of immunotherapy and chemotherapy: harnessing potential synergies. *Cancer Immunol Res* 2015; **3**: 436-443 [PMID: 25941355 DOI: 10.1158/2326-6066.CIR-15-0064]

225 **Langer CJ**, Gadgeel SM, Borghaei H, Papadimitrakopoulou VA, Patnaik A, Powell SF, Gentzler RD, Martins RG, Stevenson JP, Jalal SI, Panwalkar A, Yang JC, Gubens M, Sequist LV, Awad MM, Fiore J, Ge Y, Raftopoulos H, Gandhi L; KEYNOTE-021 investigators. Carboplatin and pemetrexed with or without pembrolizumab for advanced, non-squamous non-small-cell lung cancer: a randomised, phase 2 cohort of the open-label KEYNOTE-021 study. *Lancet Oncol* 2016; **17**: 1497-1508 [PMID: 27745820 DOI: 10.1016/S1470-2045(16)30498-3]

226 **Fuchs CS**, Doi T, Woo-Jun Jang R, Muro K, Satoh T, Machado M. KEYNOTE-059 cohort 1: Efficacy and safety of pembrolizumab (pembro) monotherapy in patients with previously treated advanced gastric cancer. *J Clin Oncol* 2017; **35**: 4003

227 **Fuchs CS**, Doi T, Jang RW, Muro K, Satoh T, Machado M, Sun W, Jalal SI, Shah MA, Metges JP, Garrido M, Golan T, Mandala M, Wainberg ZA, Catenacci DV, Ohtsu A, Shitara K, Geva R, Bleeker J, Ko AH, Ku G, Philip P, Enzinger PC, Bang YJ, Levitan D, Wang J, Rosales M, Dalal RP, Yoon HH. Safety and Efficacy of Pembrolizumab Monotherapy in Patients With Previously Treated Advanced Gastric and Gastroesophageal Junction Cancer: Phase

2 Clinical KEYNOTE-059 Trial. *JAMA Oncol* 2018; **4**: e180013 [PMID: 29543932 DOI: 10.1001/jamaoncol.2018.0013]

228 **Fuchs CS**, Ohtsu A, Tabernero J, Van Cutsem E, Wang JD, Lam B, Dalal R, Koshiji M, Banget YJ. Preliminary safety data from KEYNOTE-059: pembrolizumab plus 5-fluorouracil (5-FU) and cisplatin for first-line treatment of advanced gastric cancer. *J Clin Oncol* 2016; **34**: abstr 4037

229 **Bang YJ**, Muro K, Fuchs CS, Golan T, Geva R, Hara H, Jalal SA, Borg C, Doi T, Wainberg ZA, Wang J, Koshiji M, Dalal RP, Chung HC. KEYNOTE-059 cohort 2: safety and efficacy of pembrolizumab (pembro) plus 5-fluorouracil (5-FU) and cisplatin for first-line (1L) treatment of advanced gastric cancer. *J Clin Oncol* 2017; **35**: 4012

230 **Doi TP**, Piha-Paul SA, Jalal SI, Mai-Dang H, Saraf S, Koshiji M, Csiki I, Bennouna J. Updated results for the advanced esophageal carcinoma cohort of the phase Ib KEYNOTE-028 study of pembrolizumab (MK-3475). *J Clin Oncol* 2016; **34**: abstr 7 [DOI: 10.1200/jco.2016.34.4_suppl.7]

231 **Merck Sharp & Dohme Corp**. Study of Pembrolizumab (MK-3475) in Previously-Treated Participants With Advanced Carcinoma of the Esophagus or Esophagogastric Junction (MK-3475-180/KEYNOTE-180). [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT02559687?term=NCT02559687rank=1 ClinicalTrials.gov Identifier: NCT02559687

232 **Ohtsu A**, Tabernero J, Bang YJ, Fuchs CS, Sun L, Wang Z, Csiki I, Koshiji M, Van Cutsem E. Pembrolizumab (MK-3475) versus paclitaxel as second-line therapy for advanced gastric or gastroesophageal junction (GEJ) adenocarcinoma: phase 3 KEYNOTE-061 study. *J Clin Oncol* 2016; **34**: abstr TPS183 [DOI: 10.1200/jco.2016.34.4_suppl.tps183]

233 **Fuchs CS**, Ozguroglu M, Bang YJ, Di Bartolomeo M, Mandala M, Ryu MH, Fornaro L, Olesiński T, Caglevic C, Chung HC, Muro K, Goekkurt E, Mansoor W, McDermott RS, Shacham-Shmueli E, Chen X, Kang SP, Mayo CA, Ohtsu A, Shitara K. Pembrolizumab (pembro) vs paclitaxel (PTX) for previously treated advanced gastric or gastroesophageal junction (G/GEJ) cancer: Phase 3 KEYNOTE-061 trial. *J Clin Oncol* 2018; **36**: abstr 4062

234 **Merck Sharp Dohme Corp**. Study of Pembrolizumab (MK-3475) as First-Line Monotherapy and Combination Therapy for Treatment of Advanced Gastric or Gastroesophageal Junction Adenocarcinoma (MK-3475-062/KEYNOTE-062). [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT02494583?term=NCT02494583rank=1 ClinicalTrials.gov Identifier: NCT02494583

235 **Merck Sharp Dohme Corp**. Study of Pembrolizumab (MK-3475) Versus Investigator's Choice Standard Therapy for Participants With Advanced Esophageal/Esophagogastric Junction Carcinoma That Progressed After First-Line Therapy (MK-3475-181/KEYNOTE-181). [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT02564263?term=NCT02564263rank=1 ClinicalTrials.gov Identifier: NCT02564263

236 **Chung HC**, Arkenau H-T, Wyrwicz L, Oh DY, Lee KW, Infante JR, Lee SS, Lee J, Keilholz U, Mita AC, Plummer ER, Kemeny M, Melichar B, Smith DM, Chin KM, von Heydebreck A, Cuillerot JM, Kang YK, Safranet H. Avelumab (MSB0010718C; anti-PD-L1) in patients with advanced gastric or gastroesophageal junction cancer from JAVELIN solid tumor phase Ib trial: analysis of safety and clinical activity. *J Clin Oncol* 2016; **34**: abstr 4009

237 **Nishina T,** Shitara K, Iwasa S, Hironaka S, Muro K, Esaki T, Satoh T, Yamaguchi T, Machida N, von Heydebreck A, Watanabe M, Doi T. Safety, PD-L1 expression, and clinical activity of avelumab (MSB0010718C), an anti-PD-L1 antibody, in Japanese patients with advanced gastric or gastroesophageal junction cancer. *J Clin Oncol* 2016; **34**: abstr 168 [DOI: 10.1200/jco.2016.34.4_suppl.168]

238 **Moehler MH,** Taïeb J, Gurtler JS, Xiong H, Zhang J, Cuillerot JM, Bokuet N. Maintenance therapy with avelumab (MSB0010718C; anti-PD-L1) vs continuation of first-line chemotherapy in patients with unresectable, locally advanced or metastatic gastric cancer: the phase 3 JAVELIN Gastric 100 trial. *J Clin Oncol* 2016; **34**: TPS4134)

239 **Segal NH,** Antonia SJ, Brahmer JR, Maio M, Blake-Haskins A, Li X.,Khleif S. Preliminary data from a multi-arm expansion study of MEDI4736, an anti-PD-L1 antibody. *J Clin Oncol* 2014; **32**: 5s

240 **Kelly RJ,** Chung , Gu Y, Steele KE, Rebelatto MC, Robbins PB,Almhanna K. (2015). Phase Ib/II study to evaluate the safety and antitumor activity of durvalumab (MEDI4736) and tremelimumab as monotherapy or in combination, in patients with recurrent or metastatic gastric/gastroesophageal junction adenocarcinoma. *J Immunotherapy Cancer* 2015; **32**: P157 [DOI: 10.1186/2051-1426-3-S2-P157]

241 **Pardoll DM**. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* 2012; **12**: 252-264 [PMID: 22437870 DOI: 10.1038/nrc3239]

242 **Bristol-Myers Squibb**. An Investigational Immuno-therapy Study of Nivolumab or Placebo in Patients With Resected Esophageal or Gastroesophageal Junction Cancer (Checkmate 577). [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT02743494?term=CheckMate-577rank=1 ClinicalTrials.gov Identifier: NCT02743494

243 **Wada H.** Department of Clinical Research in Tumor Immunology, Graduate School of Medicine, Osaka University. Study of Pre-operative Combination Therapy With Mogamulizumab and Nivolumab Against Solid Cancer Patients. [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT02946671?term=NCT02946671rank=1 ClinicalTrials.gov Identifier: NCT0294667 ClinicalTrials.gov Identifier: NCT02946671

244 **European Organisation for Research and Treatment of Cancer - EORTC**. Postoperative Immunotherapy vs Standard Chemotherapy for Gastric Cancer With High Risk for Recurrence (VESTIGE). [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03443856?term=NCT03443856rank=1 ClinicalTrials.gov Identifier: NCT03443856

245 **Bristol-Myers Squibb.** Ronan K, Johns Hopkins University. Nivolumab or Nivolumab/Ipilimumab Prior to Chemoradiation Plus Nivolumab With II/Ⅲ Gastro/Esophageal Cancer. [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03044613?term=NCT03044613rank=1 ClinicalTrials.gov Identifier: NCT03044613

246 **Palta M,** Duke University. Pembrolizumab, Radiotherapy, and Chemotherapy in Neoadjuvant Treatment of Malignant Esophago-gastric Diseases (PROCEED). [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03064490?term=NCT03064490cond=gastric+cancerrank=1 ClinicalTrials.gov Identifier: NCT03064490

247 **Merck Sharp Dohme Corp**. Perioperative Chemo and Pembrolizumab in Gastric Cancer. [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: URL: https://clinicaltrials.gov/ct2/show/NCT02918162?term=pembrolizumabcond=gastric+cancerrank=4 ClinicalTrials.gov Identifier: NCT02918162

248 **Merck Sharp Dohme Corp**. Study of Pembrolizumab (MK-3475) Plus Chemotherapy Versus Placebo Plus Chemotherapy in Participants With Gastric or Gastroesophageal Junction (GEJ) Adenocarcinoma (MK-3475-585/KEYNOTE-585). [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03221426?term=KEYNOTE-585cond=gastric+cancerrank=1 ClinicalTrials.gov Identifier: NCT03221426

249 **Merck Sharp Dohme Corp**. University of Kansas Medical Center. Perioperative mFOLFOX Plus Pembrolizumab in Gastroesophageal Junction (GEJ) and Stomach Adenocarcinoma. [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/

show/NCT03488667?term=NCT03488667cond=gastric+cancerrank=1 ClinicalTrials.gov Identifier: NCT03488667

250 **Royal Marsden NHS Foundation Trust,** Merck KgaA. Study Title: Peri-operative Immuno-Chemotherapy in Operable Oesophageal and Gastric cancer (ICONIC). [accessed 2018 May 4]. In: ClinicalTrials. gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: URL: https://clinicaltrials.gov/ct2/show/NCT03399 071?term=NCT03399071cond=gastric+cancerrank=1 ClinicalTrials. gov Identifier: NCT03399071

251 **Voron T**, Marcheteau E, Pernot S, Colussi O, Tartour E, Taieb J, Terme M. Control of the immune response by pro-angiogenic factors. *Front Oncol* 2014; **4**: 70 [PMID: 24765614 DOI: 10.3389/fonc.2014.00070]

252 **Dudek AZ**, Sica A, Sidani A, Jha GG, Xie H, Alva AS, Stein MN, Singer EA. Phase Ib study of pembrolizumab in combination with bevacizumab for the treatment of metastatic renal cell carcinoma: Big Ten Cancer Research Consortium BTCRC-GU14003. *J Clin Oncol* 2016; **34**: suppl.559 [DOI: 10.1200/jco.2016.34.2_suppl.559]

253 **Solmaz S**, Johnstone PA, Forsyth PA. Safety and antitumor activity of hypofractionated stereotactic irradiation (HFSRT) with pembrolizumab (Pembro) and bevacizumab (Bev) in patients (pts) with recurrent high grade gliomas: preliminary results from phase I study. *J Clin Oncol* 2016; **34**: abstr 2041

254 **Chau I,** Calvo E, Santana-Davila R, Ahnert JR, Penel N. Interim safety and clinical activity in patients (pts) with advanced gastric or gastroesophageal junction (G/GEJ) adenocarcinoma from a multicohort phase 1 study of ramucirumab (R) plus pembrolizumab (P). *J Clin Oncol* 2017; **35**: abstract 102 [DOI: 10.1200/JCO.2017.35.4_suppl.102]

255 **Kato K,** Department of Gastrointestinal Medical Oncology, National cancer center hospital Japan. A Phase 1/2 Study of Ramucirumab Plus Nivolumab in Participants With Gastric or GEJ Cancer. [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT02999295?term=NCT02999295cond=gastric+cancerrank=1 ClinicalTrials.gov Identifier: NCT02999295

256 **St. Marianna University School of Medicine Hospital,** Department of Clinical Oncology. https://upload.umin.ac.jp/cgi-openbin/ctr_e/ctr_view.cgi?recptno=R000029783

257 **New York University School of Medicine,** National Cancer Institute (NCI). Lenvatinib Mesylate and Pembrolizumab in Treating Patients With Metastatic or Recurrent Gastric or Gastroesophageal Cancer. [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: URL: https://clinicaltrials.gov/ct2/show/NCT03413397?term=nct03413397cond=gastric+cancerrank=1 ClinicalTrials.gov Identifier: NCT03413397

258 **Institut Bergonié,** Merck KgaA. A Phase I/II Study of Regorafenib Plus Avelumab in Digestive Tumors (REGOMUNE). [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03475953?term=nct03475953cond=gastric+cancerrank=1 ClinicalTrials.gov Identifier: NCT03475953

259 **Vanneman M**, Dranoff G. Combining immunotherapy and targeted therapies in cancer treatment. *Nat Rev Cancer* 2012; **12**: 237-251 [PMID: 22437869 DOI: 10.1038/nrc3237]

260 **Catenacci DV**, Kim SS, Gold PJ. A phase 1b/2, open label, dose-escalation study of margetuximab (M) in combination with pembrolizumab (P) in patients with relapsed/refractory advanced HER2⁺ gastroesophageal (GEJ) junction or gastric (G) cancer. *J Clin Oncol* 2017; **35**: TPS219 [DOI: 10.1200/JCO.2017.35.4_suppl.TPS219]

261 **Zhai L**, Spranger S, Binder DC, Gritsina G, Lauing KL, Giles FJ, Wainwright DA. Molecular Pathways: Targeting IDO1 and Other Tryptophan Dioxygenases for Cancer Immunotherapy. *Clin Cancer Res* 2015; **21**: 5427-5433 [PMID: 26519060 DOI: 10.1158/1078-0432.CCR-15-0420]

262 **Ott PA**, Hodi FS, Kaufman HL, Wigginton JM, Wolchok JD. Combination immunotherapy: a road map. *J Immunother Cancer* 2017; **5**: 16 [PMID: 28239469 DOI: 10.1186/s40425-017-0218-5]

263 **Incyte Corporation.** Merck Sharp Dohme Corp. A Phase 1/2 Study Exploring the Safety, Tolerability, and Efficacy of Pembrolizumab (MK-3475) in Combination With Epacadostat (INCB024360) in Subjects With Selected Cancers (INCB 24360-202 / MK-3475-037 / KEYNOTE-037/ ECHO-202). [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: URL: https://clinicaltrials.gov/ct2/show/NCT02178722?term=NCT02178722rank=1 ClinicalTrials.gov Identifier: NCT02178722

264 **Kunz P,** Stanford University. Epacadostat and Pembrolizumab in Treating Patients With Metastatic or Unresectable Gastroesophageal Junction or Gastric Cancer. In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). Available from: URL: https://clinicaltrials.gov/ct2/show/NCT03196232?term=nct03196232rank=1

265 **Kidokoro T**, Tanikawa C, Furukawa Y, Katagiri T, Nakamura Y, Matsuda K. CDC20, a potential cancer therapeutic target, is negatively regulated by p53. *Oncogene* 2008; **27**: 1562-1571 [PMID: 17873905 DOI: 10.1038/sj.onc.1210799]

266 **Maire V**, Baldeyron C, Richardson M, Tesson B, Vincent-Salomon A, Gravier E, Marty-Prouvost B, De Koning L, Rigaill G, Dumont A, Gentien D, Barillot E, Roman-Roman S, Depil S, Cruzalegui F, Pierré A, Tucker GC, Dubois T. TTK/hMPS1 is an attractive therapeutic target for triple-negative breast cancer. *PLoS One* 2013; **8**: e63712 [PMID: 23700430 DOI: 10.1371/journal.pone.0063712]

267 **Yim H**. Current clinical trials with polo-like kinase 1 inhibitors in solid tumors. *Anticancer Drugs* 2013; **24**: 999-1006 [PMID: 23949254 DOI: 10.1097/CAD.0000000000000007]

268 **Gray D**, Jubb AM, Hogue D, Dowd P, Kljavin N, Yi S, Bai W, Frantz G, Zhang Z, Koeppen H, de Sauvage FJ, Davis DP. Maternal embryonic leucine zipper kinase/murine protein serine-threonine kinase 38 is a promising therapeutic target for multiple cancers. *Cancer Res* 2005; **65**: 9751-9761 [PMID: 16266996 DOI: 10.1158/0008-5472.CAN-04-4531]

269 **Wang L**, Rubinstein R, Lines JL, Wasiuk A, Ahonen C, Guo Y, Lu LF, Gondek D, Wang Y, Fava RA, Fiser A, Almo S, Noelle RJ. VISTA, a novel mouse Ig superfamily ligand that negatively regulates T cell responses. *J Exp Med* 2011; **208**: 577-592 [PMID: 21383057 DOI: 10.1084/jem.20100619]

270 **Le Mercier I**, Chen W, Lines JL, Day M, Li J, Sergent P, Noelle RJ, Wang L. VISTA Regulates the Development of Protective Antitumor Immunity. *Cancer Res* 2014; **74**: 1933-1944 [PMID: 24691994 DOI: 10.1158/0008-5472.CAN-13-1506]

271 **Liu J**, Yuan Y, Chen W, Putra J, Suriawinata AA, Schenk AD, Miller HE, Guleria I, Barth RJ, Huang YH, Wang L. Immune-checkpoint proteins VISTA and PD-1 nonredundantly regulate murine T-cell responses. *Proc Natl Acad Sci USA* 2015; **112**: 6682-6687 [PMID: 25964334 DOI: 10.1073/pnas.1420370112]

272 **Curis, Inc**. A Study of CA-170 (Oral PD-L1, PD-L2 and VISTA Checkpoint Antagonist) in Patients With Advanced Tumors and Lymphomas. [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT02812875?term=NCT02812875rank=1 ClinicalTrials.gov Identifier: NCT02812875

273 **Böger C**, Behrens HM, Krüger S, Röcken C. The novel negative checkpoint regulator VISTA is expressed in gastric carcinoma and associated with PD-L1/PD-1: A future perspective for a combined gastric cancer therapy? *Oncoimmunology* 2017; **6**: e1293215 [PMID: 28507801 DOI: 10.1080/2162402X.2017.1293215]

274 **Gilead Sciences.** Andecaliximab Combined With Nivolumab Versus Nivolumab Alone in Adults With Unresectable or Recurrent Gastric or Gastroesophageal Junction Adenocarcinoma. [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT02864381?term=NCT02864381rank=1 ClinicalTrials.gov Identifier: NCT02864381

275 **Aanur P**, Gutierrez M, Kelly RJ, Ajani JA, Ku GY, Denlinger CS. FRACTION (fast real- time assessment of combination therapies in immuno- oncology)- gastric cancer (GC): a randomized, open-label, adaptive, phase 2 study of nivolumab in combination with other

immuno- oncology (IO) agents in patients with advanced GC. *J Clin Oncol* 2017; **35**: TPS4137

276 **Mayo Clinic, National Cancer Institute (NCI)**. Pembrolizumab, Combination Chemotherapy, and Radiation Therapy Before Surgery in Treating Adult Patients With Locally Advanced Gastroesophageal Junction or Gastric Cardia Cancer That Can Be Removed by Surgery. [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT02730546?term=NCT027305 46rank=1 ClinicalTrials.gov Identifier: NCT02730546

277 **Kono K**, Fukushima Medical University Hospital. Checkpoint Inhibitor and Radiotherapy for Recurrent Gastric Cancer (CIRCUIT). [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https:// clinicaltrials.gov/ct2/show/NCT03453164?term=NCT03453164rank =1 ClinicalTrials.gov Identifier: NCT03453164

278 **Läubli H**, Balmelli C, Bossard M, Pfister O, Glatz K, Zippelius A. Acute heart failure due to autoimmune myocarditis under pembrolizumab treatment for metastatic melanoma. *J Immunother Cancer* 2015; **3**: 11 [PMID: 25901283 DOI: 10.1186/ s40425-015-0057-1]

279 **Johnson DB**, Balko JM, Compton ML, Chalkias S, Gorham J, Xu Y, Hicks M, Puzanov I, Alexander MR, Bloomer TL, Becker JR, Slosky DA, Phillips EJ, Pilkinton MA, Craig-Owens L, Kola N, Plautz G, Reshef DS, Deutsch JS, Deering RP, Olenchock BA, Lichtman AH, Roden DM, Seidman CE, Koralnik IJ, Seidman JG, Hoffman RD, Taube JM, Diaz LA Jr, Anders RA, Sosman JA, Moslehi JJ. Fulminant Myocarditis with Combination Immune Checkpoint Blockade. *N Engl J Med* 2016; **375**: 1749-1755 [PMID: 27806233 DOI: 10.1056/NEJMoa1609214]

280 **Myint ZW**, Goel G. Role of modern immunotherapy in gastrointestinal malignancies: a review of current clinical progress. *J Hematol Oncol* 2017; **10**: 86 [PMID: 28434400 DOI: 10.1186/ s13045-017-0454-7]

281 **Yonsei University, Korea**. Biomarker-Integrated Umbrella, Advanced Gastric Cancer. [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT02951091?ter m=NCT02951091rank=1 ClinicalTrials.gov Identifier: NCT0295109

282 **National Cancer Institute (NCI)**. [accessed 2018 May 4]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT 02465060?term=NCT02465060rank=1 ClinicalTrials.gov Identifier: NCT02465060

Helicobacter pylori infection and liver diseases: Epidemiology and insights into pathogenesis

Kazuya Okushin, Takeya Tsutsumi, Kazuhiko Ikeuchi, Akira Kado, Kenichiro Enooku, Hidetaka Fujinaga, Kyoji Moriya, Hiroshi Yotsuyanagi, Kazuhiko Koike

Kazuya Okushin, Akira Kado, Kenichiro Enooku, Hidetaka Fujinaga, Kazuhiko Koike, Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan

Kazuya Okushin, Kyoji Moriya, Department of Infection Control and Prevention, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan

Kazuhiko Ikeuchi, Kyoji Moriya, Department of Infectious Diseases, The University of Tokyo, Tokyo 113-8655, Japan

Takeya Tsutsumi, Kazuhiko Ikeuchi, Hiroshi Yotsuyanagi, Division of Infectious Diseases, Advanced Clinical Research Center, Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

ORCID number: Kazuya Okushin (0000-0001-9584-043X); Takeya Tsutsumi (0000-0003-0851-1887); Kazuhiko Ikeuchi (0000-0003-1677-3369); Akira Kado (0000-0003-4477-6858); Kenichiro Enooku (0000-0001-5319-8864); Hidetaka Fujinaga (0000-0003-4170-5923); Kyoji Moriya (0000-0001-9628-2724); Hiroshi Yotsuyanagi (0000-0001-7882-5262); Kazuhiko Koike (0000-0002-9739-9243).

Abstract

Both *Helicobacter pylori* (*H. pylori*) infection and liver diseases, including nonalcoholic fatty liver disease (NAFLD), viral hepatitis, and hepatocellular carcinoma (HCC), have high prevalences worldwide, and the relationship between *H. pylori* infection and liver disease has been discussed for many years. Although positive correlations between *H. pylori* and NAFLD have been identified in some clinical and experimental studies, negative correlations have also been obtained in high-quality clinical studies. Associations between *H. pylori* and the pathogenesis of chronic viral hepatitis, mainly disease progression with fibrosis, have also been suggested in some clinical studies. Concerning HCC, a possible role for *H. pylori* in hepatocarcinogenesis has been identified since *H. pylori* genes have frequently been detected in resected HCC specimens. However, no study has

revealed the direct involvement of *H. pylori* in promoting the development of HCC. Although findings regarding the correlations between *H. pylori* and liver disease pathogenesis have been accumulating, the existing data do not completely lead to an unequivocal conclusion. Further high-quality clinical and experimental analyses are necessary to evaluate the efficacy of *H. pylori* eradication in ameliorating the histopathological changes observed in each liver disease.

Key words: *Helicobacter pylori*; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Hepatitis C virus; Hepatitis B virus; Viral hepatitis; Hepatocellular carcinoma

Core tip: Both *Helicobacter pylori* (*H. pylori*) infection and liver diseases have high prevalences worldwide, and their relationship has been discussed for a long time. In this review, we comprehensively summarize positive and negative correlations suggested in clinical and experimental studies, and conclude that existing data cannot fully lead us to make a decision. We also point out the necessity of further analyses evaluating the efficacy of *H. pylori* eradication on histopathological changes in each liver disease. We believe this paper would help readers to gain a better understanding of the relationship between *H. pylori* and liver diseases.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is one of the most well-known microbes in the world. Warren and Marshall reported the possible virulence of *H. pylori* in patients with gastritis, gastric ulcer, and duodenal ulcer in 1984[1]. Approximately 50% of the global population is estimated to be infected with *H. pylori*[2], and chronic infection with *H. pylori* is one cause of chronic atrophic gastritis, peptic ulcer diseases, and gastric cancer[3,4]. Recently, findings concerning the influence of *H. pylori* on various extra-alimentary organs have accumulated[5-12]. Among these putative extra-alimentary disorders caused by *H. pylori*, the relationship with metabolic disorders remains controversial[13-23].

Liver diseases, including nonalcoholic fatty liver disease (NAFLD), chronic viral hepatitis, and hepatocellular carcinoma (HCC), also have high prevalences worldwide. Consequently, the relationship between *H. pylori* and liver diseases has been discussed and still

remains controversial[10]. Although the presence of *H. pylori* or *Helicobacter* species has been observed in liver samples from patients with various liver diseases[24-30] and findings regarding possible roles for *H. pylori* in the pathogenesis of liver diseases have been accumulating, few studies have reported a direct contribution of *H. pylori* to the pathogenesis of liver diseases. Additionally, negative correlations have been identified in high-quality clinical studies[31-34].

Currently, *H. pylori* is efficiently eradicated by various short-term treatments with combinations of antibiotics[35]. On the other hand, despite the remarkable progress in research and therapy, curative treatments have not yet been established for almost all liver diseases. Therefore, a discussion of whether *H. pylori* has a possible role in the pathogenesis of liver diseases and a clarification of the efficacy of *H. pylori* eradication in treating liver diseases are important. In this review, we present current insights into the relationship between *H. pylori* and liver diseases, such as NAFLD, chronic viral hepatitis, and HCC.

H. PYLORI AND NAFLD

NAFLD is an emerging liver disease worldwide, including in Asian countries[31,36,37]. NAFLD is a spectrum of diseases ranging from simple steatosis to nonalcoholic steatohepatitis (NASH). The latter is progressive and considered a causative factor of cirrhosis, HCC, and systemic metabolic disorders[38-40].

The initial description of NASH pathogenesis, which was previously defined as the "two-hit" theory, was presented by Day *et al*[41] in 1998 and has been discussed by other researchers. In addition to a "first-hit" of hepatic steatosis, a "second-hit," such as gut-derived endotoxins, proinflammatory cytokines, dysregulation of adipokines, oxidative stress, endoplasmic reticulum stress, and lipotoxicity, is necessary for NASH development. Considering the complicated mechanisms of NAFLD, however, the "two-hit" theory had been thought to be insufficient, and instead, the "multiple-parallel hits" hypothesis was proposed by Tilg *et al*[42]. According to this hypothesis, inflammatory mediators derived from various tissues, including the gut and adipose tissue, play a central role in the inflammatory cascade. However, the detailed pathogenesis largely remains unclear.

The relationship between *H. pylori* and NAFLD in the context of gastrointestinal tract inflammation has been long discussed but remains controversial (Table 1)[10,31-34,43-51]. Some cross-sectional or retrospective studies have not identified correlations between NAFLD and *H. pylori*[31-34]. Previously, we examined the associations of causative background factors with NAFLD by analyzing 13737 subjects in a cross-sectional study in Japan, but no correlations were observed between NAFLD and *H. pylori,* regardless of gender[31]. On the other hand, opposite results have also been reported[45-48]. In a meta-analysis, Wijarnpreecha *et al*[49] found a significantly increased risk of NAFLD among patients with *H. pylori* infection, with pooled odds ratios

Table 1 Summary of relevant studies between *Helicobacter pylori* and nonalcoholic fatty liver disease

Ref.	Year	Country	Study design	Number of subjects	Conclusion
Okushin *et al*[31]	2015	Japan	Cross-sectional study	13737	Negative
Baeg *et al*[32]	2016	South Korea	Cross-sectional study	3663	Negative
Fan *et al*[33]	2018	China	Cross-sectional study	21456	Negative
Cai *et al*[34]	2018	China	Cross-sectional study	2051	Negative
Polyzos *et al*[45]	2013	Greece	Cross-sectional study	53	Positive
Doğan *et al*[46]	2013	Turkey	Cross-sectional study	174	Positive
Kim *et al*[47]	2017	South Korea	Retrospective study	17028	Positive
Chen *et al*[48]	2017	China	Cross-sectional study	2263	Positive
Wijarnpreecha *et al*[49]	2016	Various countries	Meta-analysis	38622	Positive
Jamali *et al*[50]	2013	Iran	Prospective study (RCT)	49	Negative
Polyzos *et al*[51]	2014	Greece	Prospective study	12	Negative

RCT: Randomized controlled trial.

of 1.21 (95%CI: 1.07-1.37).

To the best of our knowledge, only two randomized prospective studies have attempted to reveal the direct correlation between *H. pylori* eradication and NAFLD. As shown in the study by Jamali *et al*[50], eradication does not exert significant effects on the liver fat content, liver function tests, lipid profiles, and homeostasis model assessment of insulin resistance (HOMA-IR) index in patients with NAFLD, although one limitation of this study was that it was conducted on dyspeptic patients with NAFLD. Polyzos *et al*[51] performed a small-scale prospective study of *H. pylori* eradication in patients with biopsy-proven NASH. In this study, eradication had no long-term effect on hepatic steatosis but showed a trend toward improving the noninvasive NAFLD fibrosis score[52]. Namely, in the *H. pylori*-eradicated group, the fibrosis scores decreased from -0.34 at baseline to -0.24 at month 12 ($P = 0.116$), whereas the scores increased in the control group from -0.38 at baseline to -0.56 at month 12 ($P = 0.249$). Larger-scale randomized prospective studies focusing on *H. pylori* eradication are needed.

NAFLD is closely related to metabolic syndrome. The relationship between *H. pylori* and metabolic syndrome has also been discussed for many years. Recently, Refaeli *et al*[53] analyzed 147936 individuals aged 25-95 years who performed the urea breath test during 2002-2012 using a large computerized database of a health maintenance organization in Israel. In this study, the prevalences of *H. pylori* infection and metabolic syndrome were 52.0% and 11.4%, respectively. Compared to noninfected patients, *H. pylori*-infected patients exhibited an increased likelihood of developing metabolic syndrome (adjusted OR: 1.15, 95%CI: 1.10-1.19). Similar results have been obtained in a meta-analysis[54], middle-sized community-based studies[55,56], and hospital-based studies[17,57,58]. On the other hand, Takeoka *et al*[59] reported unique controversial results focusing on the quantification of *H. pylori*-specific IgG concentrations. Namely, the subjects were stratified into 4 groups according to the concentration of *H. pylori*-specific IgG as follows: *H. pylori* seronegative (< 10 U/mL), low *H. pylori*-specific IgG levels (10-30 U/mL), moderate *H. pylori*-specific IgG

levels (30-50 U/mL), or high *H. pylori* -specific IgG levels (> 50 U/mL). After stratification, patients with low IgG levels had the lowest risk of metabolic syndrome, after adjusting for age, sex, smoking, drinking, and physical activity status. Using patients with the low IgG levels as the reference, patients with negative, moderate, and high IgG levels had ORs (95%CIs) of 2.15 (1.06-4.16), 3.69 (1.12-16.7), and 4.05 (1.05-26.8), respectively. Indeed, *H. pylori*-specific IgG levels do not always reflect disease severity; further discussion is needed to determine why the group with low IgG levels, but not negative for IgG, exhibited the lowest risk of metabolic syndrome. Another cross-sectional study in Japan, which analyzed 7394 cases, evaluated the correlations between *H. pylori* infection with the development of metabolic syndrome and each parameter[17]. In this study, *H. pylori* seropositivity was a significant and independent predictor of metabolic syndrome (OR: 1.39, 95%CI: 1.18-1.62, $P < 0.001$), as determined by a multivariate logistic regression analysis. Furthermore, according to the multivariate linear regression analysis, *H. pylori* seropositivity was significantly correlated with metabolic syndrome-related variables, such as higher systolic blood pressure (β coefficient = 1.03, $P = 0.014$), a lower high-density lipoprotein (HDL) cholesterol level (β coefficient = -2.00, $P < 0.001$), and a higher LDL cholesterol level (β coefficient = 2.21, $P = 0.005$). In addition, successful eradication of *H. pylori* significantly improves disturbances in these metabolic parameters[60-63]. However, some reports contradict an association between *H. pylori* and these metabolic risk factors[64-68]. Therefore, we are not able to reach a definitive conclusion, and the effect of *H. pylori* on metabolic factors may depend on the subjects examined, due to differences in factors such as country of residence, dietary habits, culture, and fitness habits.

Since obesity is closely linked to NAFLD, a relationship between *H. pylori* and obesity has also been hypothesized. A meta-analysis by Lender *et al*[69] concluded that the rates of obesity and overweight were inversely and significantly correlated with the prevalence of *H. pylori* infection ($r = 0.29$, $P < 0.001$). However, this meta-analysis only selected studies conducted in developed countries [GDP > 25000 USD/(person·year)].

Contradictory results were obtained in rather large-scale studies performed in other countries, such as China[70,71]. The reason for this discrepancy remains to be elucidated, but the difference in dietary habits and culture is probably responsible. In addition, the subjects' appetites and actual food intake levels will presumably be changed after successful eradication of *H. pylori* and may affect body weight. To determine whether the presence of *H. pylori* itself triggers body weight gain, detailed studies without exogenous factors are necessary to determine whether an *H. pylori* infection itself triggers body weight gain. Nwokolo *et al*[72] presented interesting data in a study examining this point. In a small-scale pilot trial, plasma ghrelin, leptin, and gastrin levels were measured before and after the cure of *H. pylori* in 10 subjects. After *H. pylori* cure, plasma ghrelin levels increased significantly by 75% ($P = 0.002$). On the other hand, leptin and gastrin levels have decreased by 11% and 30%, respectively, although the differences were not significant. Ghrelin is known to stimulate appetite and induce a positive energy balance, leading to body weight gain[73]; therefore, an increase in plasma ghrelin levels might be associated with the development of obesity following the eradication of *H. pylori*.

One of the important manifestations of NAFLD is insulin resistance (IR)[74]. Higher HOMA-IR scores were recorded for *H. pylori*-infected patients[55,75-78], while opposite results have also been obtained in other studies[19,79]. Cytokine production was suggested as a mechanism by which *H. pylori* induced IR. *H. pylori* infection stimulates the release of proinflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 and IL-8[80,81]. TNF-α induces IR by suppressing insulin-induced tyrosine phosphorylation of insulin receptor and its substrate, insulin receptor substrate (IRS)-1, in a hepatoma cell line[82]. In fact, neutralization of increased TNF-α levels in obese fa/fa rats significantly increases the peripheral uptake of glucose in response to insulin[83]. Adiponectin and fetuin-A are also regarded as key factors contributing to IR. Adiponectin, an adipocyte-derived hormone, antagonizes excess lipid storage in the liver and protects against inflammation and fibrosis[84]. According to Ando *et al*[85], successful eradication of *H. pylori* significantly increases total adiponectin levels from 5.61 μg/mL to 6.16 μg /mL ($P < 0.0001$) as well as the levels of each multimer form (high-, middle-, and low-molecular-weight) of adiponectin. Fetuin-A, a glycoprotein produced by the liver, is correlated with impaired insulin sensitivity, glucose metabolism, and the onset of diabetes mellitus[86,87]. *H. pylori*-positive subjects have higher fetuin-A levels and HOMA-IR scores than *H. pylori*-negative subjects. In a cross-sectional study, the mean fetuin-A values were 0.77 g/L and 0.58 g/L in *H. pylori*-positive and *H. pylori*-negative subjects, respectively. Mean HOMA-IR scores were 3.1 and 2.2 in *H. pylori*-positive and *H. pylori*-negative subjects, respectively. In addition, a significant positive correlation between fetuin-A and HOMA-IR was observed after adjusting for other factors (adjusted coefficient $\beta = 0.23$, $P < 0.01$)[88].

Based on these results, levels of inflammatory cytokines, adiponectin and fetuin-A may be associated with *H. pylori*-related IR, although that relationship has not been completely acknowledged.

Recently, the gut microbiota has been the focus of studies on the pathogenesis of various diseases and has also been suggested to play key roles in NAFLD pathogenesis[89,90]. Cytotoxin-associated gene A antigen (CagA), the known virulence factor of *H. pylori*, has been reported to alter the gut microbiota, resulting in the exacerbation of cell proliferation and immune phenotypes[91]. Furthermore, increased mucosal permeability of the intestine induced by *H. pylori* infection was reported[92]. These alterations in the gut environment, such as the microbiota and mucosal barrier, by *H. pylori* may influence the pathogenesis of NAFLD.

In summary, positive correlations between *H. pylori* and NAFLD have been reported in some clinical and experimental studies, but other studies have presented contradictory data. Further analyses focusing on the effect of *H. pylori* eradication on histopathological changes in patients with biopsy-proven NAFLD are necessary.

H. PYLORI AND CHRONIC VIRAL HEPATITIS OR CIRRHOSIS

The involvement of *H. pylori* in the pathogenesis of chronic viral hepatitis has been speculated (Table 2). Esmat *et al*[30] evaluated the presence of the *H. pylori* CagA gene in liver samples from patients with hepatitis C virus (HCV)-related chronic hepatitis or cirrhosis by the polymerase chain reaction (PCR). In this study, the *H. pylori* gene was detected in 28.2% cases of late fibrosis (F3 + F4) and 5.9% cases of early fibrosis (F1 + F2) ($P = 0.0001$) by PCR. The influence of *H. pylori* on the progression of HCV-related liver diseases has also been examined. Anti-*H. pylori* antibody positivity was significantly and independently associated with cirrhosis in patients with HCV-related chronic hepatitis or cirrhosis in multivariate analyses (OR: 2.42, 95%CI: 1.06-5.53, $P = 0.037$)[93]. Rocha *et al*[94]. examined liver tissues from *H. pylori*-infected patients and revealed that the *Helicobacter* 16S rDNA was only detected in 4.2% of liver samples from control patients and in 3.5% of samples from patients with noncirrhotic chronic hepatitis C. The *Helicobacter* 16S rDNA was detected in 68.0% of liver samples from patients with HCV-positive cirrhosis without HCC as well as in 61.3% of patients with HCC. In a meta-analysis, Wang *et al*[95] analyzed the prevalence of *H. pylori* infection in a total of 1449 patients with chronic hepatitis C and 2377 control cases. The prevalence of *H. pylori* was significantly higher in patients with chronic hepatitis C than in those without chronic hepatitis C (pooled odds ratio 2.93). In a subgroup analysis, the odds ratios were 4.48 for HCV-related cirrhosis and 5.45 for HCC. These results suggest an association between *Helicobacter* species and HCV-related disease progression, but these findings only show the presence of *H. pylori*, not its pathogenicity

Table 2 Summary of relevant studies between *Helicobacter pylori* and chronic viral hepatitis, cirrhosis, and hepatocellular carcinoma

Ref.	Year	Country	Study design	Number of subjects	Conclusion
HCV					
Esmat *et al*[30]	2012	Egypt	Cross-sectional study	85	Positive
Queiroz *et al*[93]	2006	Argentina	Cross-sectional study	106	Positive
Rocha *et al*[94]	2005	France	Cross-sectional study	109	Positive
Wang *et al*[95]	2016	Various countries	Meta-analysis	3826	Positive
HBV					
Ponzetto *et al*[96]	2000	Italy	Case-control study	355	Positive
Huang *et al*[97]	2017	China	Cross-sectional study	608	Positive
Mohamed *et al*[98]	2018	Egypt	Cross-sectional study	170	Positive
Wang *et al*[99]	2011	China	Cross-sectional study	1872	Negative
Wang *et al*[100]	2016	China	Meta-analysis	4645	Positive
HCC					
Nilsson *et al*[24]	2001	Sweden	Cross-sectional study	36	Positive
Pellicano *et al*[25]	2004	Italy	Cross-sectional study	26	Positive
Huang *et al*[26]	2004	China	Cross-sectional study	36	Positive
Xuan *et al*[27]	2006	China	Cross-sectional study	50	Positive
Xuan *et al*[101]	2008	Various countries	Meta-analysis	522	Positive

HCV: Hepatitis C virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma.

in the liver. *H. pylori* may putatively flow into the liver *via* the portal vein and be caught and eliminated by intrahepatic immune cells such as Kupffer cells in the normal liver. Since the number of these cells decreases with the progression of fibrosis, *H. pylori* is speculated to be present in the liver as a result of immune escape. Accordingly, the prevalence of *H. pylori* might simply be high in patients with cirrhosis compared with that in control or noncirrhotic patients. Additional in-depth studies are required to confirm the actual involvement of *H. pylori* in the progression of liver fibrosis.

Clinical findings suggesting relationships between *H. pylori* and hepatitis B virus (HBV)-related liver diseases have also been reported[96-100]. A higher prevalence of *H. pylori* infection in HBV-infected patients has been reported in several studies[96-98], but these findings may just reflect the hygienic environments in childhood. Actually, Wang *et al*[99] reported that the prevalence of *H. pylori* infection in asymptomatic HBV carriers was 38.67% in Shandong Province, China, which was not different than that in the normal adult population recruited from the same region (35.94%, *P* = 0.352). Possible associations between the progression of HBV-related liver disease and liver-related complications, such as variceal bleeding, ascites, and encephalopathy, have also been reported[97]. In a meta-analysis of a Chinese population, the prevalence of *H. pylori* infection among patients with HBV-related liver diseases increased as the disease severity increased[100]. Namely, the *H. pylori*-positive rate in patients with chronic hepatitis B patients but not cirrhosis or HCC was 2.44-fold higher than that in healthy controls (pooled OR: 2.44, 95%CI: 1.85-3.24; *P* < 0.01). Furthermore, the *H. pylori*-positive rate in patients with HBV-induced cirrhosis was 4.28-fold higher (pooled OR: 4.28, 95%CI: 2.99-6.13, *P* < 0.01) than that in healthy controls, while it was 6.02-fold higher (pooled OR: 6.02, 95%CI: 4.33-8.37, *P* = 0.821) in patients with HBV-related HCC. Therefore, the presence of *H. pylori* may accelerate the progression of HBV-related liver

pathogenesis, but the precise pathogenicity in the liver remains to be elucidated.

In summary, although *H. pylori* infection and chronic viral hepatitis seem to be associated in limited situations, further studies are necessary to obtain a final conclusion since researchers have not yet clearly determined whether *H. pylori* itself directly contributes to the progression of viral hepatitis.

H. PYLORI AND HEPATOCARCINOGENESIS

Several clinical studies have reported an association between *H. pylori* and HCC (Table 2). *H. pylori* and similar species were detected in liver samples from patients with HCC[24-27]. Additionally, a positive association between *H. pylori* and the risk of HCC was reported in a meta-analysis[101]. The overall prevalence of *H. pylori* in the liver was 53.3% (129 of 242) in patients with HCC and 10.4% (29 of 280) in controls, and the odds ratio for the association between *H. pylori* infection and the risk of HCC was 13.63 (95%CI: 7.90-23.49). These observations, however, only showed the presence of *H. pylori* in liver tissues. HCC is usually accompanied by liver fibrosis, and in these circumstances, the intrahepatic immune status and hemodynamics may be changed to permit the inflow of *H. pylori* and escape from immunity in the liver, as noted above. Therefore, the presence of *H. pylori* only in HCC tissues does not provide strong support for an association with HCC.

Some *in vitro* studies have presented the possible mechanism underlying the association between *H. pylori* and hepatocarcinogenesis. As shown in the study by Zhang *et al*[102], *H. pylori* causes pathological effects on HepG2 hepatoma cells by upregulating the expression of some proteins related to gene transcription and signal transduction. Virulent type *H. pylori* cause cell cycle arrest and apoptosis of Huh7 cells, another hepatoma cell line[103]. According to Liu *et al*[104], histidine-rich protein

(Hpn), a small histidine-rich cytoplasmic protein from *H. pylori*, induces apoptosis by suppressing ubiquitin-specific peptidase 5 (USP5) expressions and activating the P14-P53 signaling pathway. However, these data are only indirect findings obtained from *in vitro* studies using cancer cell lines. Indeed, to the best of our knowledge, direct evidence for the tumorigenic effect of *H. pylori* on the liver has not been obtained. Ki *et al*[105] postulated that *H. pylori* infection might promote the transforming growth factor (TGF)-β1-dependent oncogenic pathway, disturbing the balance between hepatocyte apoptosis and proliferation in a murine model of CCl_4-induced fibrosis, but in this study, the development of HCC itself was not observed. Furthermore, in transgenic mice expressing HCV proteins, *H. pylori* infection did not promote the development of HCC[106]. Based on these findings, *H. pylori* infection is currently presumed to be unlikely to contribute to HCC development.

In summary, although *H. pylori* genes are frequently detected in HCC samples, possible correlations between *H. pylori* and hepatocarcinogenesis seem to be doubtful. Further studies showing the direct contribution *in vivo* using infectious animal models or mice transgenic for *H. pylori* genes are necessary to confirm this relationship.

CONCLUSION

H. pylori have a high prevalence, and its roles in liver diseases, as well as its well-known contribution to the pathogenesis of gastric disorders, have been discussed. As described in this review, several correlations between *H. pylori* and liver diseases, particularly NAFLD, have been reported in some clinical and experimental studies, but these correlations remain controversial. Further analyses are required to elucidate the associations. In addition, since only a few studies have examined the effect of *H. pylori* eradication on the pathogenesis of NAFLD, histopathological confirmation that *H. pylori* eradication specifically prevents or improves disease progression is necessary. Concerning chronic viral hepatitis and HCC, some observational studies suggested positive correlations. But, we have to recognize possibilities of the publication bias and confounding factors such as hygienic environments and contaminations resulting from the presence of cirrhosis. Actually, few studies have definitively confirmed the pathogenic contribution of *H. pylori* to increase of inflammation, progression of fibrosis, or acceleration of hepatocarcinogenesis. *H. pylori* infection and liver diseases still have high prevalences worldwide and significant impact on patients' prognosis. There is a room to discuss whether *H. pylori* are really involved in pathogenesis of each liver disease. To demonstrate the actual involvement of *H. pylori* in these processes, *H. pylori* itself or its gene product must be shown to accelerate the pathogenesis of these diseases using well-established animal models.

ACKNOWLEDGMENTS

REFERENCES

1   **Marshall BJ**, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; **1**: 1311-1315 [PMID: 6145023 DOI: 10.1016/S0140-6736(84)91816-6]

2   **Cover TL**, Blaser MJ. Helicobacter pylori in health and disease. *Gastroenterology* 2009; **136**: 1863-1873 [PMID: 19457415 DOI: 10.1053/j.gastro.2009.01.073]

3   **Malfertheiner P**, Megraud F, O'Morain CA, Atherton J, Axon AT, Bazzoli F, Gensini GF, Gisbert JP, Graham DY, Rokkas T, El-Omar EM, Kuipers EJ; European Helicobacter Study Group. Management of Helicobacter pylori infection--the Maastricht IV/ Florence Consensus Report. *Gut* 2012; **61**: 646-664 [PMID: 22491499 DOI: 10.1136/gutjnl-2012-302084]

4   **Matsuhisa T**, Aftab H. Observation of gastric mucosa in Bangladesh, the country with the lowest incidence of gastric cancer, and Japan, the country with the highest incidence. *Helicobacter* 2012; **17**: 396-401 [PMID: 22967124 DOI: 10.1111/j.1523-5378.2012.00967.x]

5   **Patel P**, Mendall MA, Carrington D, Strachan DP, Leatham E, Molineaux N, Levy J, Blakeston C, Seymour CA, Camm AJ. Association of Helicobacter pylori and Chlamydia pneumoniae infections with coronary heart disease and cardiovascular risk factors. *BMJ* 1995; **311**: 711-714 [PMID: 7549683 DOI: 10.1136/bmj.311.7007.711]

6   **Federman DG**, Kirsner RS, Moriarty JP, Concato J. The effect of antibiotic therapy for patients infected with Helicobacter pylori who have chronic urticaria. *J Am Acad Dermatol* 2003; **49**: 861-864 [PMID: 14576665 DOI: 10.1016/S0190-9622(03)00846-6]

7   **Maurer KJ**, Ihrig MM, Rogers AB, Ng V, Bouchard G, Leonard MR, Carey MC, Fox JG. Identification of cholelithogenic enterohepatic helicobacter species and their role in murine cholesterol gallstone formation. *Gastroenterology* 2005; **128**: 1023-1033 [PMID: 15825083 DOI: 10.1053/j.gastro.2005.01.008]

8   **Takahashi Y**, Yamamichi N, Shimamoto T, Mochizuki S, Fujishiro M, Takeuchi C, Sakaguchi Y, Niimi K, Ono S, Kodashima S, Mitsushima T, Koike K. Helicobacter pylori infection is positively associated with gallstones: a large-scale cross-sectional study in Japan. *J Gastroenterol* 2014; **49**: 882-889 [PMID: 23736795 DOI: 10.1007/s00535-013-0832-z]

9   **Franceschi F**, Zuccalà G, Roccarina D, Gasbarrini A. Clinical effects of Helicobacter pylori outside the stomach. *Nat Rev Gastroenterol Hepatol* 2014; **11**: 234-242 [PMID: 24345888 DOI: 10.1038/nrgastro.2013.243]

10 **Waluga M**, Kukla M, Żorniak M, Bacik A, Kotulski R. From the stomach to other organs: Helicobacter pylori and the liver. *World J Hepatol* 2015; **7**: 2136-2146 [PMID: 26328025 DOI: 10.4254/wjh.v7.i18.2136]

11 **Rabelo-Gonçalves EM**, Roesler BM, Zeitune JM. Extragastric manifestations of Helicobacter pylori infection: Possible role of bacterium in liver and pancreas diseases. *World J Hepatol* 2015; **7**: 2968-2979 [PMID: 26730276 DOI: 10.4254/wjh.v7.i30.2968]

12 **de Korwin JD**, Ianiro G, Gibiino G, Gasbarrini A. Helicobacter pylori infection and extragastric diseases in 2017. *Helicobacter* 2017; **22** Suppl 1 [PMID: 28891133 DOI: 10.1111/hel.12411]

13 **Pietroiusti A**, Diomedi M, Silvestrini M, Cupini LM, Luzzi I, Gomez-Miguel MJ, Bergamaschi A, Magrini A, Carrabs T, Vellini M, Galante A. Cytotoxin-associated gene-A--positive Helicobacter pylori strains are associated with atherosclerotic stroke. *Circulation* 2002; **106**: 580-584 [PMID: 12147540 DOI: 10.1161/01.CIR.0000023894.10871.2F]

14 **Diomedi M**, Pietroiusti A, Silvestrini M, Rizzato B, Cupini LM, Ferrante F, Magrini A, Bergamaschi A, Galante A, Bernardi G. CagA-positive Helicobacter pylori strains may influence the natural history of atherosclerotic stroke. *Neurology* 2004; **63**: 800-804 [PMID: 15365126 DOI: 10.1212/01.WNL.0000138025.82419.80]

15 **Cho I**, Blaser MJ, François F, Mathew JP, Ye XY, Goldberg JD, Bini EJ. Helicobacter pylori and overweight status in the United States: data from the Third National Health and Nutrition Examination Survey. *Am*

J Epidemiol 2005; **162**: 579-584 [PMID: 16093294 DOI: 10.1093/aje/kwi237]

16   **Longo-Mbenza B**, Nkondi Nsenga J, Vangu Ngoma D. Prevention of the metabolic syndrome insulin resistance and the atherosclerotic diseases in Africans infected by Helicobacter pylori infection and treated by antibiotics. *Int J Cardiol* 2007; **121**: 229-238 [PMID: 17368586 DOI: 10.1016/j.ijcard.2006.12.003]

17   **Gunji T**, Matsuhashi N, Sato H, Fujibayashi K, Okumura M, Sasabe N, Urabe A. Helicobacter pylori infection is significantly associated with metabolic syndrome in the Japanese population. *Am J Gastroenterol* 2008; **103**: 3005-3010 [PMID: 19086952 DOI: 10.1111/j.1572-0241.2008.02151.x]

18   **Satoh H**, Saijo Y, Yoshioka E, Tsutsui H. Helicobacter Pylori infection is a significant risk for modified lipid profile in Japanese male subjects. *J Atheroscler Thromb* 2010; **17**: 1041-1048 [PMID: 20610892 DOI: 10.5551/jat.5157]

19   **Naja F**, Nasreddine L, Hwalla N, Moghames P, Shoaib H, Fatfat M, Sibai A, Gali-Muhtasib H. Association of H. pylori infection with insulin resistance and metabolic syndrome among Lebanese adults. *Helicobacter* 2012; **17**: 444-451 [PMID: 23066847 DOI: 10.1111/j.1523-5378.2012.00970.x]

20   **Stergiopoulos C**, Kountouras J, Daskalopoulou-Vlachoyianni E, Polyzos SA, Zavos C, Vlachoyiannis E, Kokkali S, Deretzi G, Kapetanakis N, Katsinelos P, Gavalas E. Helicobacter pylori may play a role in both obstructive sleep apnea and metabolic syndrome. *Sleep Med* 2012; **13**: 212-213 [PMID: 22137108 DOI: 10.1016/j.sleep.2011.04.016]

21   **Shin DW**, Kwon HT, Kang JM, Park JH, Choi HC, Park MS, Park SM, Son KY, Cho B. Association between metabolic syndrome and Helicobacter pylori infection diagnosed by histologic status and serological status. *J Clin Gastroenterol* 2012; **46**: 840-845 [PMID: 23064216 DOI: 10.1097/MCG.0b013e3182522477]

22   **Buzás GM**. Metabolic consequences of Helicobacter pylori infection and eradication. *World J Gastroenterol* 2014; **20**: 5226-5234 [PMID: 24833852 DOI: 10.3748/wjg.v20.i18.5226]

23   **Lee M**, Baek H, Park JS, Kim S, Kyung C, Baik SJ, Lee BK, Kim JH, Ahn CW, Kim KR, Kang S. Current Helicobacter pylori infection is significantly associated with subclinical coronary atherosclerosis in healthy subjects: A cross-sectional study. *PLoS One* 2018; **13**: e0193646 [PMID: 29499055 DOI: 10.1371/journal.pone.0193646]

24   **Nilsson HO**, Mulchandani R, Tranberg KG, Stenram U, Wadström T. Helicobacter species identified in liver from patients with cholangiocarcinoma and hepatocellular carcinoma. *Gastroenterology* 2001; **120**: 323-324 [PMID: 11246512 DOI: 10.1053/gast.2001.21382]

25   **Pellicano R**, Mazzaferro V, Grigioni WF, Cutufia MA, Fagoonee S, Silengo L, Rizzetto M, Ponzetto A. Helicobacter species sequences in liver samples from patients with and without hepatocellular carcinoma. *World J Gastroenterol* 2004; **10**: 598-601 [PMID: 14966925 DOI: 10.3748/wjg.v10.i4.598]

26   **Huang Y**, Fan XG, Wang ZM, Zhou JH, Tian XF, Li N. Identification of helicobacter species in human liver samples from patients with primary hepatocellular carcinoma. *J Clin Pathol* 2004; **57**: 1273-1277 [PMID: 15563667 DOI: 10.1136/jcp.2004.018556]

27   **Xuan SY**, Li N, Qiang X, Zhou RR, Shi YX, Jiang WJ. Helicobacter infection in hepatocellular carcinoma tissue. *World J Gastroenterol* 2006; **12**: 2335-2340 [PMID: 16688821 DOI: 10.3748/wjg.v12.i15.2335]

28   **Cindoruk M**, Cirak MY, Unal S, Karakan T, Erkan G, Engin D, Dumlu S, Turet S. Identification of Helicobacter species by 16S rDNA PCR and sequence analysis in human liver samples from patients with various etiologies of benign liver diseases. *Eur J Gastroenterol Hepatol* 2008; **20**: 33-36 [PMID: 18090988 DOI: 10.1097/MEG.0b013e3282efa4f2]

29   **Pirouz T**, Zounubi L, Keivani H, Rakhshani N, Hormazdi M. Detection of Helicobacter pylori in paraffin-embedded specimens from patients with chronic liver diseases, using the amplification method. *Dig Dis Sci* 2009; **54**: 1456-1459 [PMID: 18975076 DOI: 10.1007/s10620-008-0522-5]

30   **Esmat G**, El-Bendary M, Zakarya S, Ela MA, Zalata K. Role of Helicobacter pylori in patients with HCV-related chronic hepatitis and cirrhosis with or without hepatocellular carcinoma: possible association with disease progression. *J Viral Hepat* 2012; **19**: 473-479 [PMID: 22676359 DOI: 10.1111/j.1365-2893.2011.01567.x]

31   **Okushin K**, Takahashi Y, Yamamichi N, Shimamoto T, Enooku K, Fujinaga H, Tsutsumi T, Shintani Y, Sakaguchi Y, Ono S, Kodashima S, Fujishiro M, Moriya K, Yotsuyanagi H, Mitsushima T, Koike K. Helicobacter pylori infection is not associated with fatty liver disease including non-alcoholic fatty liver disease: a large-scale cross-sectional study in Japan. *BMC Gastroenterol* 2015; **15**: 25 [PMID: 25880912 DOI: 10.1186/s12876-015-0247-9]

32   **Baeg MK**, Yoon SK, Ko SH, Noh YS, Lee IS, Choi MG. Helicobacter pylori infection is not associated with nonalcoholic fatty liver disease. *World J Gastroenterol* 2016; **22**: 2592-2600 [PMID: 26937147 DOI: 10.3748/wjg.v22.i8.2592]

33   **Fan N**, Peng L, Xia Z, Zhang L, Wang Y, Peng Y. *Helicobacter pylori* Infection Is Not Associated with Non-alcoholic Fatty Liver Disease: A Cross-Sectional Study in China. *Front Microbiol* 2018; **9**: 73 [PMID: 29445363 DOI: 10.3389/fmicb.2018.00073]

34   **Cai O**, Huang Z, Li M, Zhang C, Xi F, Tan S. Association between *Helicobacter pylori* Infection and Nonalcoholic Fatty Liver Disease: A Single-Center Clinical Study. *Gastroenterol Res Pract* 2018; **2018**: 8040262 [PMID: 29527224 DOI: 10.1155/2018/8040262]

35   **Suzuki H**, Mori H. World trends for H. pylori eradication therapy and gastric cancer prevention strategy by H. pylori test-and-treat. *J Gastroenterol* 2018; **53**: 354-361 [PMID: 29138921 DOI: 10.1007/s00535-017-1407-1]

36   **Kojima S**, Watanabe N, Numata M, Ogawa T, Matsuzaki S. Increase in the prevalence of fatty liver in Japan over the past 12 years: analysis of clinical background. *J Gastroenterol* 2003; **38**: 954-961 [PMID: 14614602 DOI: 10.1007/s00535-003-1178-8]

37   **Satapathy SK**, Sanyal AJ. Epidemiology and Natural History of Nonalcoholic Fatty Liver Disease. *Semin Liver Dis* 2015; **35**: 221-235 [PMID: 26378640 DOI: 10.1055/s-0035-1562943]

38   **Marrero JA**, Fontana RJ, Su GL, Conjeevaram HS, Emick DM, Lok AS. NAFLD may be a common underlying liver disease in patients with hepatocellular carcinoma in the United States. *Hepatology* 2002; **36**: 1349-1354 [PMID: 12447858 DOI: 10.1053/jhep.2002.36939]

39   **Rubinstein E**, Lavine JE, Schwimmer JB. Hepatic, cardiovascular, and endocrine outcomes of the histological subphenotypes of nonalcoholic fatty liver disease. *Semin Liver Dis* 2008; **28**: 380-385 [PMID: 18956294 DOI: 10.1055/s-0028-1091982]

40   **Baffy G**, Brunt EM, Caldwell SH. Hepatocellular carcinoma in non-alcoholic fatty liver disease: an emerging menace. *J Hepatol* 2012; **56**: 1384-1391 [PMID: 22326465 DOI: 10.1016/j.jhep.2011.10.027]

41   **Day CP**, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998; **114**: 842-845 [PMID: 9547102 DOI: 10.1016/S0016-5085(98)70599-2]

42   **Tilg H**, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. *Hepatology* 2010; **52**: 1836-1846 [PMID: 21038418 DOI: 10.1002/hep.24001]

43   **Tang DM**, Kumar S. The Association Between Helicobacter pylori Infection and Nonalcoholic Fatty Liver Disease. *Curr Gastroenterol Rep* 2017; **19**: 5 [PMID: 28155087 DOI: 10.1007/s11894-017-0545-1]

44   **Cheng DD**, He C, Ai HH, Huang Y, Lu NH. The Possible Role of *Helicobacter pylori* Infection in Non-alcoholic Fatty Liver Disease. *Front Microbiol* 2017; **8**: 743 [PMID: 28539915 DOI: 10.3389/fmicb.2017.00743]

45   **Polyzos SA**, Kountouras J, Papatheodorou A, Patsiaoura K, Katsiki E, Zafeiriadou E, Zavos C, Anastasiadou K, Terpos E. Helicobacter pylori infection in patients with nonalcoholic fatty liver disease. *Metabolism* 2013; **62**: 121-126 [PMID: 22841522 DOI: 10.1016/j.metabol.2012.06.007]

46   **Doğan Z**, Filik L, Ergül B, Sarikaya M, Akbal E. Association between Helicobacter pylori and liver-to-spleen ratio: a randomized-controlled single-blind study. *Eur J Gastroenterol Hepatol* 2013; **25**: 107-110 [PMID: 23013624 DOI: 10.1097/MEG.0b013e3283590c10]

47   **Kim TJ**, Sinn DH, Min YW, Son HJ, Kim JJ, Chang Y, Baek SY, Ahn SH, Lee H, Ryu S. A cohort study on Helicobacter pylori infection associated with nonalcoholic fatty liver disease. *J Gastroenterol* 2017; **52**: 1201-1210 [PMID: 28382402 DOI: 10.1007/s00535-017-1337-y]

48   **Chen CX**, Mao YS, Foster P, Zhu ZW, Du J, Guo CY. Possible association between Helicobacter pylori infection and nonalcoholic fatty liver disease. *Appl Physiol Nutr Metab* 2017; **42**: 295-301 [PMID: 28177748 DOI: 10.1139/apnm-2016-0499]

49   **Wijarnpreecha K**, Thongprayoon C, Panjawatanan P, Manatsathit W, Jaruvongvanich V, Ungprasert P. Helicobacter pylori and Risk of Nonalcoholic Fatty Liver Disease: A Systematic Review and Meta-analysis. *J Clin Gastroenterol* 2018; **52**: 386-391 [PMID: 28098578 DOI: 10.1097/MCG.0000000000000784]

50   **Jamali R**, Mofid A, Vahedi H, Farzaneh R, Dowlatshahi S. The effect of helicobacter pylori eradication on liver fat content in subjects with non-alcoholic Fatty liver disease: a randomized open-label clinical trial. *Hepat Mon* 2013; **13**: e14679 [PMID: 24358044 DOI: 10.5812/hepatmon.14679]

51   **Polyzos SA**, Nikolopoulos P, Stogianni A, Romiopoulos I, Katsinelos P, Kountouras J. Effect of Helicobacter pylori eradication on hepatic steatosis, NAFLD fibrosis score and HSENSI in patients with nonalcoholic steatohepatitis: a MR imaging-based pilot open-label study. *Arq Gastroenterol* 2014; **51**: 261-268 [PMID: 25296089 DOI: 10.1590/S0004-28032014000300017]

52   **Angulo P**, Hui JM, Marchesini G, Bugianesi E, George J, Farrell GC, Enders F, Saksena S, Burt AD, Bida JP, Lindor K, Sanderson SO, Lenzi M, Adams LA, Kench J, Therneau TM, Day CP. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. *Hepatology* 2007; **45**: 846-854 [PMID: 17393509 DOI: 10.1002/hep.21496]

53   **Refaeli R**, Chodick G, Haj S, Goren S, Shalev V, Muhsen K. Relationships of H. pylori infection and its related gastroduodenal morbidity with metabolic syndrome: a large cross-sectional study. *Sci Rep* 2018; **8**: 4088 [PMID: 29511278 DOI: 10.1038/s41598-018-22198-9]

54   **Upala S**, Jaruvongvanich V, Riangwiwat T, Jaruvongvanich S, Sanguankeo A. Association between Helicobacter pylori infection and metabolic syndrome: a systematic review and meta-analysis. *J Dig Dis* 2016; **17**: 433-440 [PMID: 27273478 DOI: 10.1111/1751-2980.12367]

55   **Chen LW**, Chien CY, Yang KJ, Kuo SF, Chen CH, Chien RN. Helicobacter pylori Infection Increases Insulin Resistance and Metabolic Syndrome in Residents Younger than 50 Years Old: A Community-Based Study. *PLoS One* 2015; **10**: e0128671 [PMID: 26020514 DOI: 10.1371/journal.pone.0128671]

56   **Chen LW**, Chien CY, Hsieh CW, Chang LC, Huang MH, Huang WY, Kuo SF, Chien CH, Lin CL, Chien RN. The Associations Between Helicobacter pylori Infection, Serum Vitamin D, and Metabolic Syndrome: A Community-Based Study. *Medicine* (Baltimore) 2016; **95**: e3616 [PMID: 27149497 DOI: 10.1097/MD.0000000000003616]

57   **Chen TP**, Hung HF, Chen MK, Lai HH, Hsu WF, Huang KC, Yang KC. Helicobacter Pylori Infection is Positively Associated with Metabolic Syndrome in Taiwanese Adults: a Cross-Sectional Study. *Helicobacter* 2015; **20**: 184-191 [PMID: 25582223 DOI: 10.1111/hel.12190]

58   **Yang W**, Xuan C. Influence of Helicobacter pylori Infection on Metabolic Syndrome in Old Chinese People. *Gastroenterol Res Pract* 2016; **2016**: 6951264 [PMID: 27429613 DOI: 10.1155/2016/6951264]

59   **Takeoka A**, Tayama J, Yamasaki H, Kobayashi M, Ogawa S, Saigo T, Hayashida M, Shirabe S. Impact of Helicobacter pylori Immunoglobulin G Levels and Atrophic Gastritis Status on Risk of Metabolic Syndrome. *PLoS One* 2016; **11**: e0166588 [PMID: 27851820 DOI: 10.1371/journal.pone.0166588]

60   **Scharnagl H**, Kist M, Grawitz AB, Koenig W, Wieland H, März W. Effect of Helicobacter pylori eradication on high-density lipoprotein cholesterol. *Am J Cardiol* 2004; **93**: 219-220 [PMID: 14715353 DOI: 10.1016/j.amjcard.2003.09.045]

61   **Ando T**, Minami M, Ishiguro K, Maeda O, Watanabe O, Mizuno T, Fujita T, Takahashi H, Noshiro M, Goto H. Changes in biochemical parameters related to atherosclerosis after Helicobacter pylori eradication. *Aliment Pharm Therap* 2006; **24**: 58-64 [DOI: 10.1111/j.1365-2036.2006.00026.x]

62   **Gen R**, Demir M, Ataseven H. Effect of Helicobacter pylori eradication on insulin resistance, serum lipids and low-grade inflammation. *South Med J* 2010; **103**: 190-196 [PMID: 20134372 DOI: 10.1097/SMJ.0b013e3181cf373f]

63   **Mokhtare M**, Mirfakhraee H, Arshad M, Samadani Fard SH, Bahardoust M, Movahed A, Masoodi M. The effects of helicobacter pylori eradication on modification of metabolic syndrome parameters in patients with functional dyspepsia. *Diabetes Metab Syndr* 2017; **11** Suppl 2: S1031-S1035 [PMID: 28780229 DOI: 10.1016/j.dsx.2017.07.035]

64   **Woodward M**, Morrison C, McColl K. An investigation into factors associated with Helicobacter pylori infection. *J Clin Epidemiol* 2000; **53**: 175-181 [PMID: 10729690 DOI: 10.1016/S0895-4356(99)00171-7]

65   **Gillum RF**. Infection with Helicobacter pylori, coronary heart disease, cardiovascular risk factors, and systemic inflammation: the Third National Health and Nutrition Examination Survey. *J Natl Med Assoc* 2004; **96**: 1470-1476 [PMID: 15586651]

66   **Mostaza JM**, Camino N, Gerique JG, Peña R, Baquero M, Lahoz C. C-reactive protein levels and prevalence of chronic infections in subjects with hypoalphalipoproteinemia. *Metabolism* 2005; **54**: 33-37 [PMID: 15562377 DOI: 10.1016/j.metabol.2004.07.009]

67   **Sotiropoulos A**, Gikas A, Skourtis S, Merkouris P, Pentzeridis P, Polydorou A, Pappas S. Seropositivity to Chlamydia pneumoniae or Helicobacter pylori and coronary artery disease. *Int J Cardiol* 2006; **109**: 420-421 [PMID: 15993502 DOI: 10.1016/j.ijcard.2005.05.039]

68   **Alzahrani S**, Nelson J, Moss SF, Paulus JK, Knowler WC, Pittas AG; Diabetes Prevention Program Research Group. H. pylori seroprevalence and risk of diabetes: An ancillary case-control study nested in the diabetes prevention program. *J Diabetes Complications* 2017; **31**: 1515-1520 [PMID: 28739267 DOI: 10.1016/j.jdiacomp.2017.05.015]

69   **Lender N**, Talley NJ, Enck P, Haag S, Zipfel S, Morrison M, Holtmann GJ. Review article: Associations between Helicobacter pylori and obesity--an ecological study. *Aliment Pharmacol Ther* 2014; **40**: 24-31 [PMID: 24832176 DOI: 10.1111/apt.12790]

70   **Xu C**, Yan M, Sun Y, Joo J, Wan X, Yu C, Wang Q, Shen C, Chen P, Li Y, Coleman WG Jr. Prevalence of Helicobacter pylori infection and its relation with body mass index in a Chinese population. *Helicobacter* 2014; **19**: 437-442 [PMID: 25256639 DOI: 10.1111/hel.12153]

71   **Zhang Y**, Du T, Chen X, Yu X, Tu L, Zhang C. Association between Helicobacter pylori infection and overweight or obesity in a Chinese population. *J Infect Dev Ctries* 2015; **9**: 945-953 [PMID: 26409735 DOI: 10.3855/jidc.6035]

72   **Nwokolo CU**, Freshwater DA, O'Hare P, Randeva HS. Plasma ghrelin following cure of Helicobacter pylori. *Gut* 2003; **52**: 637-640 [PMID: 12692045 DOI: 10.1136/gut.52.5.637]

73   **Inui A**, Asakawa A, Bowers CY, Mantovani G, Laviano A, Meguid MM, Fujimiya M. Ghrelin, appetite, and gastric motility: the emerging role of the stomach as an endocrine organ. *FASEB J* 2004; **18**: 439-456 [PMID: 15003990 DOI: 10.1096/fj.03-0641rev]

74   **Chitturi S**, Abeygunasekera S, Farrell GC, Holmes-Walker J, Hui JM, Fung C, Karim R, Lin R, Samarasinghe D, Liddle C, Weltman M, George J. NASH and insulin resistance: Insulin hypersecretion and specific association with the insulin resistance syndrome. *Hepatology* 2002; **35**: 373-379 [PMID: 11826411 DOI: 10.1053/jhep.2002.30692]

75   **Eshraghian A**, Hashemi SA, Hamidian Jahromi A, Eshraghian H, Masoompour SM, Davarpanah MA, Eshraghian K, Taghavi SA. Helicobacter pylori infection as a risk factor for insulin resistance. *Dig Dis Sci* 2009; **54**: 1966-1970 [PMID: 19009348 DOI: 10.1007/s10620-008-0557-7]

76   **Gunji T**, Matsuhashi N, Sato H, Fujibayashi K, Okumura M, Sasabe N, Urabe A. Helicobacter pylori infection significantly increases insulin resistance in the asymptomatic Japanese population. *Helicobacter* 2009; **14**: 144-150 [PMID: 19751440 DOI: 10.1111/j.1523-5378.2009.00705.x]

77   **Polyzos SA**, Kountouras J, Zavos C, Deretzi G. The association between Helicobacter pylori infection and insulin resistance: a systematic review. *Helicobacter* 2011; **16**: 79-88 [PMID: 21435084 DOI: 10.1111/j.1523-5378.2011.00822.x]

78   **Polyzos SA**, Kountouras J, Zavos C, Deretzi G. Helicobacter pylori Infection and insulin resistance. *Helicobacter* 2013; **18**: 165-166 [PMID: 23066701 DOI: 10.1111/hel.12019]

79   **Sumida Y**, Kanemasa K, Imai S, Mori K, Tanaka S, Shimokobe H, Kitamura Y, Fukumoto K, Kakutani A, Ohno T, Taketani H, Seko Y, Ishiba H, Hara T, Okajima A, Yamaguchi K, Moriguchi M, Mitsuyoshi H, Yasui K, Minami M, Itoh Y. Helicobacter pylori infection might have a potential role in hepatocyte ballooning in nonalcoholic fatty liver disease. *J Gastroenterol* 2015; **50**: 996-1004 [PMID: 25622927 DOI: 10.1007/s00535-015-1039-2]

80   **Peek RM Jr**, Blaser MJ. Helicobacter pylori and gastrointestinal tract adenocarcinomas. *Nat Rev Cancer* 2002; **2**: 28-37 [PMID: 11902583 DOI: 10.1038/nrc703]

81   **Basso D**, Plebani M, Kusters JG. Pathogenesis of Helicobacter pylori infection. *Helicobacter* 2010; **15** Suppl 1: 14-20 [PMID: 21054648 DOI: 10.1111/j.1523-5378.2010.00781.x]

82   **Feinstein R**, Kanety H, Papa MZ, Lunenfeld B, Karasik A. Tumor necrosis factor-alpha suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. *J Biol Chem* 1993; **268**: 26055-26058 [PMID: 8253716]

83   **Hotamisligil GS**, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 1993; **259**: 87-91 [PMID: 7678183 DOI: 10.1126/science.7678183]

84   **Buechler C**, Wanninger J, Neumeier M. Adiponectin, a key adipokine in obesity related liver diseases. *World J Gastroenterol* 2011; **17**: 2801-2811 [PMID: 21734787]

85   **Ando T**, Ishikawa T, Takagi T, Imamoto E, Kishimoto E, Okajima A, Uchiyama K, Handa O, Yagi N, Kokura S, Naito Y, Mizuno S, Asakawa A, Inui A, Yoshikawa T. Impact of Helicobacter pylori eradication on circulating adiponectin in humans. *Helicobacter* 2013; **18**: 158-164 [PMID: 23167259 DOI: 10.1111/hel.12028]

86   **Stefan N**, Fritsche A, Weikert C, Boeing H, Joost HG, Häring HU, Schulze MB. Plasma fetuin-A levels and the risk of type 2 diabetes. *Diabetes* 2008; **57**: 2762-2767 [PMID: 18633113 DOI: 10.2337/db08-0538]

87   **Kantartzis K**, Machann J, Schick F, Fritsche A, Häring HU, Stefan N. The impact of liver fat vs visceral fat in determining categories of prediabetes. *Diabetologia* 2010; **53**: 882-889 [PMID: 20099057 DOI: 10.1007/s00125-010-1663-6]

88   **Manolakis AC**, Tiaka EK, Kapsoritakis AN, Georgoulias P, Tsiopoulos F, Valotassiou V, Potamianos SP. Increased fetuin A levels in Helicobacter pylori infection: a missing link between H. pylori and insulin resistance? *Diabetologia* 2011; **54**: 472-474 [PMID: 21116603 DOI: 10.1007/s00125-010-1995-2]

89   **Henao-Mejia J**, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, Thaiss CA, Kau AL, Eisenbarth SC, Jurczak MJ, Camporez JP, Shulman GI, Gordon JI, Hoffman HM, Flavell RA. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. *Nature* 2012; **482**: 179-185 [PMID: 22297845 DOI: 10.1038/nature10809]

90   **Quigley EM**, Abu-Shanab A, Murphy EF, Stanton C, Monsour HP Jr. The Metabolic Role of the Microbiome: Implications for NAFLD and the Metabolic Syndrome. *Semin Liver Dis* 2016; **36**: 312-316 [PMID: 27997970 DOI: 10.1055/s-0036-1593880]

91   **Jones TA**, Hernandez DZ, Wong ZC, Wandler AM, Guillemin K. The bacterial virulence factor CagA induces microbial dysbiosis that contributes to excessive epithelial cell proliferation in the Drosophila gut. *PLoS Pathog* 2017; **13**: e1006631 [PMID: 29049360 DOI: 10.1371/journal.ppat.1006631]

92   **Fukuda Y**, Bamba H, Okui M, Tamura K, Tanida N, Satomi M, Shimoyama T, Nishigami T. Helicobacter pylori infection increases mucosal permeability of the stomach and intestine. *Digestion* 2001; **63** Suppl 1: 93-96 [PMID: 11173917 DOI: 10.1159/000051918]

93   **Queiroz DM**, Rocha AM, Rocha GA, Cinque SM, Oliveira AG, Godoy A, Tanno H. Association between Helicobacter pylori infection and cirrhosis in patients with chronic hepatitis C virus. *Dig Dis Sci* 2006; **51**: 370-373 [PMID: 16534683 DOI: 10.1007/s10620-006-3150-y]

94   **Rocha M**, Avenaud P, Ménard A, Le Bail B, Balabaud C, Bioulac-Sage P, de Magalhães Queiroz DM, Mégraud F. Association of Helicobacter species with hepatitis C cirrhosis with or without hepatocellular carcinoma. *Gut* 2005; **54**: 396-401 [PMID: 15710989 DOI: 10.1136/gut.2004.042168]

95   **Wang J**, Li WT, Zheng YX, Zhao SS, Li N, Huang Y, Zhou RR, Huang ZB, Fan XG. The Association between Helicobacter pylori Infection and Chronic Hepatitis C: A Meta-Analysis and Trial Sequential Analysis. *Gastroenterol Res Pract* 2016; **2016**: 8780695 [PMID: 26904112 DOI: 10.1155/2016/8780695]

96   **Ponzetto A**, Pellicano R, Leone N, Berrutti M, Turrini F, Rizzetto M. *Helicobacter pylori* seroprevalence in cirrhotic patients with hepatitis B virus infection. *Neth J Med* 2000; **56**: 206-210 [PMID: 10821975 DOI: 10.1016/S0300-2977(00)00033-4]

97   **Huang J**, Cui J. Evaluation of Helicobacter pylori Infection in Patients with Chronic Hepatic Disease. *Chin Med J* (Engl) 2017; **130**: 149-154 [PMID: 28091405]

98   **Mohamed AA**, Elshimy AA, El Sadik AO, Ezzat E, Nasar M, Elshaer SSM, Sayed MM. Association between Severity of Liver Disease, Frequency of Helicobacter pylori Infection, and Degree of Gastric Lesion in Egyptian Patients with Hepatitis B Virus Infection. *Am J Trop Med Hyg* 2018; **98**: 221-226 [PMID: 29342404 DOI: 10.4269/ajtmh.17-0291]

99   **Wang MY**, Yue JY, Zhang YX, Liu XD, Gao XZ. Helicobacter pylori infection in asymptomatic HBV carriers, alcohol users and normal adult population in Shandong Province, China. *Clin Res Hepatol Gastroenterol* 2011; **35**: 560-562 [PMID: 21680277 DOI: 10.1016/j.clinre.2010.12.014]

100   **Wang J**, Chen RC, Zheng YX, Zhao SS, Li N, Zhou RR, Huang Y, Huang ZB, Fan XG. Helicobacter pylori infection may increase the risk of progression of chronic hepatitis B disease among the Chinese population: a meta-analysis. *Int J Infect Dis* 2016; **50**: 30-37 [PMID: 27457918 DOI: 10.1016/j.ijid.2016.07.014]

101   **Xuan SY**, Xin YN, Chen AJ, Dong QJ, Qiang X, Li N, Zheng MH, Guan HS. Association between the presence of H pylori in the liver and hepatocellular carcinoma: a meta-analysis. *World J Gastroenterol* 2008; **14**: 307-312 [PMID: 18186573 DOI: 10.3748/wjg.14.307]

102   **Zhang Y**, Fan XG, Chen R, Xiao ZQ, Feng XP, Tian XF, Chen ZH. Comparative proteome analysis of untreated and Helicobacter pylori-treated HepG2. *World J Gastroenterol* 2005; **11**: 3485-3489 [PMID: 15948260 DOI: 10.3748/wjg.v11.i22.3485]

103   **Ito K**, Yamaoka Y, Yoffe B, Graham DY. Disturbance of apoptosis and DNA synthesis by Helicobacter pylori infection of hepatocytes. *Dig Dis Sci* 2008; **53**: 2532-2540 [PMID: 18253829 DOI: 10.1007/s10620-007-0163-0]

104   **Liu Y**, Wang WM, Zou LY, Li L, Feng L, Pan MZ, Lv MY, Cao Y, Wang H, Kung HF, Pang JX, Fu WM, Zhang JF. Ubiquitin specific peptidase 5 mediates Histidine-rich protein Hpn induced cell apoptosis in hepatocellular carcinoma through P14-P53 signaling. *Proteomics* 2017; **17**: [PMID: 28523650 DOI: 10.1002/pmic.201600350]

105   **Ki MR**, Goo MJ, Park JK, Hong IH, Ji AR, Han SY, You SY, Lee EM, Kim AY, Park SJ, Lee HJ, Kim SY, Jeong KS. Helicobacter pylori accelerates hepatic fibrosis by sensitizing transforming growth factor-β1-induced inflammatory signaling. *Lab Invest* 2010; **90**: 1507-1516 [PMID: 20531291 DOI: 10.1038/labinvest.2010.109]

106   **García A**, Feng Y, Parry NM, McCabe A, Mobley MW, Lertpiriyapong K, Whary MT, Fox JG. Helicobacter pylori infection does not promote hepatocellular cancer in a transgenic mouse model of hepatitis C virus pathogenesis. *Gut Microbes* 2013; **4**: 577-590 [PMID: 23929035 DOI: 10.4161/gmic.26042]

MINIREVIEWS

Expansion of the hepatocellular carcinoma Milan criteria in liver transplantation: Future directions

Mihai-Calin Pavel, Josep Fuster

Mihai-Calin Pavel, Josep Fuster, HepatoBilioPancreatic Surgery and Transplant Unit, Department of Surgery, Digestive and Metabolic Diseases Institute, Hospital Clínic, University of Barcelona, Barcelona, Catalonia 08036, Spain

Josep Fuster, Barcelona-Clínic Liver Cancer Group (BCLC), Liver Unit, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), CIBEREHD, Universitat de Barcelona, Barcelona, Catalonia 08036, Spain

ORCID number: Mihai-Calin Pavel (0000-0003-2370-6842); Josep Fuster (0000-0002-1713-4262).

Abstract

Milan criteria are currently the benchmark related to liver transplantation (LT) for hepatocellular carcinoma. However, several groups have proposed different expanded criteria with acceptable results. In this article, we review the current status of LT beyond the Milan criteria in three different scenarios-expanded criteria with cadaveric LT, downstaging to Milan criteria before LT, and expansion in the context of adult living donor LT. The review focuses on three main questions: what would the impact of the expansion beyond Milan criteria be on the patients on the waiting list; whether the dichotomous criteria (yes/no) currently used are appropriate for LT or continuous survival estimations, such as the one of "Metroticket" and whether it should enter into the clinical practice; and, whether the use of living donor LT in the context of expansion beyond Milan criteria is justified.

Key words: Hepatocellular carcinoma; Milan criteria; Liver transplantation; Living donor liver transplantation; Expanded criteria; Downstaging

Core tip: After more than 20 years since their first description, the Milan criteria still represent the benchmark in liver transplantation for hepatocellular carcinoma. This review focuses on three unresolved issues, those being: the impact of expansion beyond Milan criteria for patients on the liver transplant waiting list; whether the dichotomous criteria (yes/no) currently used are appropriate for liver transplantation or continuous survival estimations, such as the one of "Metroticket" and whether

it should enter into the clinical practice; and, whether the use of living donor liver transplantation in the context of expansion beyond Milan criteria is justified.

INTRODUCTION

Nowadays, hepatocellular carcinoma (HCC) represents the second cause of cancer-related death in the world[1]. Liver transplantation (LT) is an attractive option for treatment of HCC, giving that it simultaneously addresses the HCC and the cirrhotic liver, which is at risk for development of new tumors.

Since the introduction of the so-called Milan criteria (MC; single lesion $\leqslant$ 5 cm or up to three separate lesions, none larger than 3 cm)[2] into clinical use, survival rates after LT for HCC have improved significantly. Today, the 5-year overall survival (OS) of patients within the MC reaches similar rates as those of nontumoral indications (65%-70% for HCC patients)[3,4]. As a result, the MC have been included in the Barcelona-Clínic Liver Cancer (BCLC) pretransplant staging, and the American Association for the Study of Liver Diseases (AASLD) and the European Association for the Study of the Liver-European Organisation for Research and Treatment of Cancer (EASL-EORTC) practice guidelines[5-8].

However, the MC may seem too restrictive. Several groups have proposed different expansions of these classic criteria, with reasonable life expectancy after LT[9-14]. The rationale behind the expansion is that approximately 25% of the patients classified as Milan-in before LT present a Milan-out HCC in the explant histology[2,15,16]. The 5-year OS of these patients are better than the minimum acceptable rate of 50% proposed by some authors[17,18]. Despite this, the majority of the transplant centers are still using the MC. Currently, the EASL-EORTC guidelines on management of the HCC do not recommend the expansion of criteria outside of prospective research studies[8].

The International Consensus Conference regarding LT for HCC that was held in Zurich in 2010 states that the MC represent the benchmark for selection of patients for transplantation and the basis for comparison with other suggested criteria. However, according to the same group, modest expansion may be considered giving the favorable results of several studies[19]. Theoretically speaking, at least three different scenarios may be planned for the expansion of the HCC criteria - transplantation with deceased donor grafts in Milan-out HCC patients, living donor (LD)LT for patients beyond MC, and successful downstaging to MC before LT in patients initially Milan-out. Regarding the first of these three scenarios, the definition of a "time 0" (the moment when the patients with expanded criteria are included on the waiting list) will be very important, allowing for study of the expansion from an "intention-to-treat" point of view[20].

Several important issues should be discussed in order to evaluate the impact of the expansion beyond MC.

The first issue to be considered is what the effect of transplanting Milan-out patients on the waiting list for LT will be[21], by balancing the survival benefit for the patients beyond MC against the harm caused by delaying the LT for the other patients on the waiting list. According to the data published by United Network for Organ Sharing (known as UNOS) and the European Liver Transplant Registry (known as ELTR), the most important problem facing LT remains the scarcity of donors[3,4]. Theoretically, the expansion of criteria could lead to an overload of an already-large waiting list by adding patients that, until this moment, were deemed to not benefit from this treatment. The decision on whether to expand the criteria depends on what number would be considered as acceptable lowest survival after LT by each transplant community[22].

The second issue to be considered is if the decision to transplant an HCC patient should only depend on rigid criteria, like "size and number" or should be a dynamic decision in which expansion beyond the MC could be an option, depending on other characteristics such as the waiting list times and donor availability in each geographic region that performs LT.

The third issue to be considered is the strategy of using LDLT in patients with HCC, which is still questioned by some authors. Despite the advantage of transplanting patients beyond MC without affecting the conventional waiting list, at least two important problems have to be analyzed. One of them is the risk to the donor, especially in the context of expanding the criteria. The second one is that there are reports that describe significantly worse results with LDLT, as compared with conventional LT[23].

The objectives of this article are to review the current literature related to the expansion beyond MC in the three described scenarios and to evaluate the relevant data linked to the issues presented above. We believe that the transplant with deceased donor grafts and the LDLT are marked by different characteristics, therefore we will discuss each one separately.

LT with deceased donor grafts in patients with HCC beyond MC

In the last years, it has become evident that the conventional LT (with cadaveric donors) for HCC beyond MC is not necessarily associated with worse results. Several authors have described modest expansions of the MC with acceptable OS and recurrence rates (see Tables 1 and 2). Giving all these results, Mazzaferro[24] suggests that the tumor size and number used as criteria for transplantation should be defined at a regional

Table 1 Expanded criteria used for liver transplantation

Criteria	Type of donor	Detailed criteria
UCSF[9,27]	Cadaveric	Solitary tumor ≤ 6.5 cm or ≤ 3 tumors with the largest ≤ 4.5 cm
Up-to-seven[10]	Cadaveric/LDLT	Seven: sum of tumor number and size of the largest tumor without microvascular invasion
Clinica Universidad de Navara (CUN)[12]	Cadaveric	1 tumor ≤ 6 cm or ≤ 3 tumors with the largest ≤ 5 cm
Toso[29]	Cadaveric	Total tumor volume ≤ 115 cm³ and AFP ≤ 400 ng/mL
Hangzhou University[13]	Cadaveric	One of the following:
		Total tumor diameter ≤ 8 cm
		Total tumor diameter > 8 cm with histological grade Ⅰ or Ⅱ and AFP ≤ 400 ng/mL
Onaca (ITR)[32]	Cadaveric	Solitary tumor, ≤ 6 cm
		2-4 tumors, ≤ 5 cm
Tokyo (5-5 rule)[53]	LDLT	Maximum 5 tumors ≤ 5 cm
Kyoto[55]	LDLT	≤ 10 tumors, ≤ 5 cm,
		DCP§ ≤ 400 mAU/mL
Kyushu University[57]	LDLT	Any number of tumors with diameter ≤ 5 cm or DCP§ ≤ 300 mAU/mL
Asan[58]	LDLT	≤ 6 tumors, diameter ≤ 5 cm
Samsung[59]	LDLT/cadaveric	≤ 7 tumors, diameter ≤ 6 cm, AFP ≤ 1000 ng/mL
BCLC[14]	LDLT	1 tumor, ≤ 7 cm
		3 tumors, ≤ 5 cm
		5 tumors, ≤ 3 cm
		Maintained response within Milan criteria during 6 mo after downstaging

AFP: Alpha-fetoprotein; BCLC: Barcelona-Clínic Liver Cancer; DCP: Des-gamma-carboxy prothrombin; LDLT: Living donor liver transplantation; LT: Liver transplantation.

Table 2 Results after liver transplantation with expanded criteria

Ref.	Type	Patients, *n* (type)	Criteria (findings)	Survival, time (%)	Recurrence, time (%)	Factors for survival	Factors for recurrence
Yao *et al*[9], 2001	R	14 (MO)	UCSF (Histol)	5 yr (84.6)	-	pT4, total tumor diameter	-
Yao *et al*[27], 2007	P	38 (MO)	UCSF (Radiol)		5 yr DFS (93.6)		UCSF Vascular invasion AFP > 1000 ng/mL
Onaca *et al*[32], 2007	R	129 (MO)	Onaca		5 yr DFS (63.9)		Tumor > 6 cm AFP > 200 ng/mL Tumors > 4
Herrero *et al*[28], 2008	P	26 (MO)	CUN (Radiol)	5 yr (73) 5 yr I-to-T (68)			Vascular invasion
Zheng *et al*[13], 2008	R	99 (MI and MO), 26 (MO)	Hangzhou (Histol)	5 yr (70.7)	5 yr DFS (62.4)	Macrovascular invasion Tumor size > 8 cm AFP > 400 ng/mL Histological grading (Ⅲ)	Macrovascular invasion Tumor size > 8 cm AFP > 400 ng/mL Histological grading (Ⅲ)
Mazzaferro *et al*[10], 2009	R	283 (MI and MO)	Up-to-seven (Histol)	5 yr (71.2)	-	Microvascular invasion Tumor grade	-
Toso *et al*[29], 2015	P	38 (MO)	Toso (Radiol)	4 yr (74.6) 4 yr I-to-T (53.8)	4 yr DFS (68)	-	-
Togashi *et al*[54], 2016	R	14 (MO)	Tokyo	-	5 yr (8)	-	Tokyo criteria AFP ≥ 400 ng/mL DCP ≥ 200 mAU/mL
Kaido *et al*[56], 2013	R	42 (MO)	Kyoto	5 yr (80)	5 yr (7)		Kyoto criteria Pretreatment of the HCC
Shirabe *et al*[57], 2011	R	48 (MI and MO)	Kyushu (Histol)		5 yr DFS (80)		Kyushu criteria
Lee *et al*[58], 2008	R	174 (MI and MO)	Asan (Histol)	5 yr (81.6)	5 yr (15)	Largest tumor > 5 cm Number > 6 Gross vascular invasion	Largest tumor > 5 cm Number > 6 Gross vascular invasion
Kim *et al*[59], 2014	R	180 (in the whole study, including Samsung-out)	Samsung (Histol)		5 yr DFS -89.6		Tumors ≤ 7 Diameter ≤ 6 cm AFP ≤ 1000 ng/mL
Llovet *et al*[14], 2018	P	22	BCLC (Radiol)	5 yr (80.2)	5 yr (23.8)	MI after locoregional therapies	

AFP: Alpha-fetoprotein; BCLC: Barcelona-Clínic Liver Cancer; DFS: Disease-free survival; Histol: Histology; I-to-T: Intention-to-treat; LT: Liver transplantation; MI: Milan-in; MO: Milan-out; P: Prospective; R: Retrospective; Radiol: Radiology; UCSF: University of California San Francisco.

level depending on the dynamics of the waiting list, the proportion of patients with and without HCC on the waiting list, the harm to the patients remaining on the waiting list, and the donor availability.

The San Francisco group published, in 2001, an expansion based on explant histological characteristics (solitary tumor ≤ 6.5 cm or up to three tumors ≤ 4.5 cm)[9]. The reported 5-year OS was 75.2% for all the patients meeting the University of California San Francisco (UCSF) criteria (including Milan-in) and was 84.6% for the 14 patients classified as Milan-out UCSF-in. However, it is expected that the pretransplantation radiological evaluation underestimates, with up to 25%-30% for the HCC stage, when it is compared to posttransplant histology findings[25,26]. For this reason, the same group published, 6 years later, the results of a prospective study using the same criteria applied to the pretransplant radiology exam. The 5-year disease-free survival (DFS) was of 91.1% for Milan-in patients *vs* 93.6% for Milan-out UCSF-in patients[27]. However, the application of these criteria was questioned by other authors. Decaens *et al*[20] analyzed the results of the UCSF criteria according to the intention-to-treat principle in a group with a relatively reduced waiting list time, of only 4 mo. When the UCSF criteria were applied at the "time 0" of inclusion on the waiting list, the 5-year OS of the Milan-out UCSF-in patients was 45.6% and of the Milan-in patients was 60.1%.

In 2009, Mazzaferro *et al*[10] published the results of a large, multicentric, retrospective study and identified a combination of tumor maximum size and number of nodules as a predictive factor for survival. The "up-to-seven" criteria (see Table 1) in patients without microvascular invasion was found to be associated with 5-year OS rate of 71.2%, which was comparable with that of the Milan-in patients. However, when the up-to-seven criteria was associated with microvascular invasion, the survival was significantly worse (48.1%). It is important to mention that the presence of microvascular invasion represents a variable not possible to identify before LT and that expansion beyond the MC is usually associated with higher rates of microvascular invasion[20].

The group of Pamplona, Spain reported the results of LT with the Clinic of Universidad of Navarra (CUN) criteria[12,28]. The 5-year OS was 68% when the analysis was performed from an intention-to-treat point of view, being statistically comparable to that for the patients with Milan-in tumors. Although none of the patients with Milan-out CUN-in HCC developed tumor recurrence in the posttransplant follow-up period, 12 of the patients recruited for that study progressed beyond the CUN criteria on the waiting list and were deemed to not benefit from LT[28].

Toso *et al*[29] published the results of a prospective study with criteria which included total tumor volume and alpha-fetoprotein (AFP). Survival and recurrence rates of the Milan-out patients meeting the criteria were acceptable, even though the "intention-to-treat

analysis" showed statistically inferior results due to the waiting list drop-out rates. The criteria of the University of Hangzhou, China also took AFP levels into account[13]. Two conclusions could be drawn from that study: first, the application of this criteria did not yield worse results when compared with MC; second, even the patients exceeding the MC but fulfilling the Hangzhou criteria presented improved prognosis when compared with the Hangzhou-out patients. It has to be mentioned that, currently, the AFP level is included in the selection criteria in France and Canada, where patients with values ≥ 1000 ng/dL are excluded for LT[30,31].

Onaca *et al*[32] analyzed the results of the International Registry of Hepatic Tumors in Liver Transplantation and concluded, similarly, that a modest expansion beyond MC could still offer favorable results (see Table 1). When patients presented in the explant analysis with one tumor of ≤ 6 cm or 2-4 tumors of ≤ 5 cm, the 5-year DFS was 64%.

Downstaging to Milan-in HCC before LT
In the context of HCC, there is a clear difference between the "bridge treatments" (referring to patients already on the waiting list for LT and submitted to locoregional therapies in order to diminish the drop-out rates) and the "downstaging" (defined as the treatment applied to patients initially outside of the established criteria). The latter is mainly used as a selection tool for the patients with better prognosis that could benefit from LT[33]. The strategy of downstaging to MC before LT by using locoregional therapies has been the subject of debate. In this review we will only be referring to the prospective studies related to the subject.

Roayaie *et al*[11] describes the results of the protocol of Mount Sinai Medical Center, which consisted of arterial chemoembolization with mitomycin C, doxorubicin and cisplatin at the time of diagnosis, LT with single systemic intraoperative dose of doxorubicin before revascularization of the new liver, and systemic doxorubicin for a total of six cycles, beginning on the sixth postoperative week. This protocol was applied to patients with unresectable HCC larger than 5 cm. The 5-year DFS of a subgroup of patients with tumors of 5-7 cm was considered acceptable (55%).

Yao *et al*[34] published, in 2015, an intention-to-treat study for a group of patients transplanted after downstaging and compared their results with the ones of Milan-in patients from an intention-to-treat point of view. Even though the cumulative risk for drop-out was higher in the downstage group (34.2% *vs* 25.6% at 2 years), the 5-year OS and the 5-year intention-to-treat OS were not statistically different between the groups. The factors related to the probability of drop-out were AFP > 1000 ng/mL and cirrhosis of Child B grade.

The group of Bologna also compared the results of downstaging and LT in 48 patients with those of 129 Milan-in patients, and concluded that the rates of transplantation, DFS and intention-to-treat OS were

Table 3 Prospective studies of downstaging of hepatocellular carcinoma before liver transplantation

Ref.	Criteria	Downstaging success rate (%)	LT rate (%)	Survival, time and rate (%)	HCC recurrence (%)
Roayaie *et al*[11], 2002	Mount Sinai protocol		53.75	5 yr OS (44) 5 yr DFS (48) 5 yr DFS, tumors < 7 cm (55)	
Yao *et al*[72], 2015	Beyond Milan: single tumor ⩽ 8 cm, 2–3 tumors (at least one > 3 and ⩽ 5 cm, total diameter ⩽ 8 cm), 4–5 tumors each ⩽ 3 cm and total diameter ⩽ 8 cm	To MC: 65.3	54.2	5 yr OS (77.8) 5 yr I-to-T (56.1)	7.8
Bologna criteria - Ravaioli *et al*[35], 2008	Beyond Milan: 1 lesion ⩽ 6 cm, 2 lesions ⩽ 5 cm, 3–5 lesions ⩽ 4 cm and total diameter ⩽ 12 cm	To MC: 72.9	66.7	3 yr DFS (71) 3 yr I-to-T (56.3)	18.8
Millonig *et al*[73], 2007	UCSF	RECIST	84.8	5 yr CR (66.6); PR (63.7); NR (25)	25
Graziadei *et al*[37], 2003	Beyond Milan, no upper limit	Partial response (> 50% of tumor size)	66.6	4 yr OS (41); 5 yr I-to-T (31)	30

CR: Complete response; HCC: Hepatocellular carcinoma; I-to-T: Intention-to-treat; LT: Liver transplantation; NR: No response; PR: Partial response; UCSF: University of California San Francisco.

comparable between the two groups[35]. On the other hand, Millonig *et al*[36] studied the effect of transarterial chemoembolization (TACE) on Milan-in and Milan-out UCSF-in patients. The response-to-treatment was evaluated according to RECIST criteria. Better intention-to treat OS and OS rates were observed for the Milan-in patients with complete or partial response to the treatment. Interestingly, the association of good response to TACE and good prognosis was not observed in the Milan-out patients, who were also more likely to drop-out or to present with recurrence after LT.

Graziadei *et al*[37] published the results of a series of HCC patients without pretreatment criteria, with the only criteria for transplantability being a response of 50% or more of the total tumoral volume. With this type of protocol, the results were statistically inferior to those of Milan-in patients submitted to the same therapy (see Table 3).

Overall, the results of these studies and several other retrospective studies are positive and offer the possibility of identifying a group of patients that can obtain acceptable survival rates after LT, despite presenting with a tumoral stage beyond MC. The EASL-EORTC guidelines of 2012 did not recommend the downstaging outside of prospective trials[8], but the AASLD guidelines of 2018 not only recommend locoregional therapies for the Milan-in patients on the waiting list but also suggest that the patients beyond the MC should be considered for LT after successful downstaging to Milan[38] when this status is maintained at least 3 mo to 6 mo[39]. However, the level of evidence and the strength of the recommendation are still very low, probably because of the lack of intention-to-treat studies related to downstaging in the literature[39]. The same type of recommendation related to LT after successful downstaging has been included in the EASL Clinical Practice Guidelines of 2018[40].

Effect of expanding beyond the MC on the LT waiting list
The main problem facing LT remains the difference between the availability of organs and the number of patients on the waiting list. The last Organ Procurement and Transplantation Network (commonly known as OPTN) report[3], from 2012, describes an increase of the median pretransplant waiting time from 12.9 mo in 2009 to 18.5 mo in 2011. Similar data have been published by the European LT Registry[4]. It seems clear that by expanding the HCC transplant criteria, the number of possible candidates on the waiting list will rise. The two main questions related to expanding beyond the MC are: what is the minimal acceptable OS after LT for HCC patients; and, whether the expansion beyond MC would have a positive or a negative effect on the posttransplant survival of all the patients on the waiting list.

Initial reports suggested 50% as the minimal acceptable survival after LT for HCC patients[41], but the International Consensus Conference Report for LT for HCC from Zurich 2012 reported that the expansion beyond MC has to take into account the effect of delaying the LT for all potential liver recipients on the waiting list, including the ones with non-tumoral indications[19]. Therefore, this report recommends to reserve LT for patients who have an expected survival comparable to that of non-HCC patients.

Using a theoretical Markov model, the group from Michigan, United States compared the survival benefit of transplanting a patient with an HCC beyond the MC and the harm caused to the other patients on the waiting list[21]. The results of that study showed that the adoption of more liberal criteria would lead to an increase in risk of death (of 44%) among all patients on the waiting list. The adverse effect caused by expanding the criteria would outweigh its benefits when the expected 5-year OS of the transplanted Milan-out patients would be of

less than 61%. However, this result was very sensitive to the characteristics of donation and waiting list times of each geographical region, offering values between 25% and 72%.

Ten years after that publication, the analysis could be very different. Graft characteristics will have changed, with increased use of expanded criteria donors, such as aged donors, steatotic livers or donation after cardiac death (DCD) grafts[42]. On the other hand, factors related with the recipient's prognosis, like administration of direct-acting antiviral (DAA) treatment with 90% rates of hepatitis C virus (HCV) negativization, could change the characteristics of the waiting list[43]. In the last reports of the United States' transplant registry, the HCV was no longer the principal indication for LT, being overcome by HCC and alcohol intake[44]. Furthermore, recent published data have shown continuous improvement of the post-transplantation survival rates[44,45].

Related to the use of expanded criteria donors in LT for HCC, a theoretical model study from the University of Chicago, United States, from 2012, compared the effectiveness of DCD *vs* brain-dead donor LT in terms of costs, quality of life and beyond 1-year survival[46]. In the context of HCC, the use of DCD livers for LT, when compared with the alternative of waiting for a brain-dead donor liver, resulted in a survival benefit for patients without model for end-stage liver disease (commonly known as MELD) prioritization points. However, that study only referred to Milan-in patients. The inclusion of patients beyond the MC onto the waiting list could change the results of this analysis.

As described above, modest expansion of the HCC LT indications may offer results comparable to those of Milan-in patients and of non-HCC recipients. Since several expansion studies reported 5-year survival rates of more than 70%, it seems that LT can be an option for carefully selected patients beyond MC.

A different approach to separate the patients with good or bad prognosis after LT, including those beyond MC, would be the use of combined scores which take into account tumor characteristics (total tumor volume, rather than size and number) and AFP cut-off values (see above)[29,40,47]. In this way, both large HCC and small ones with potentially aggressive behavior as well as poor post-LT outcomes could be identified.

Regarding the effect of expanding criteria for the waiting list, the analysis is more complex, taking into account not only the recipient prognosis but also characteristics that depend of each geographic region that performs LT, like the number of patients on the waiting list, available donors, and their quality. Thus, the decision on whether to expand the HCC transplantation criteria should probably be made at a regional level after analyzing the impact of all these items.

Dichotomous *vs* continuous selection criteria
Despite the success of the MC in LT for HCC, one of the questions that has arisen is whether a dichotomous yes/no criteria is the best strategy to decide which patients should benefit from the transplant. Even inside the MC, there is a 10%-15% risk of recurrence after LT[48] and, as discussed above, several expanded criteria of LT are associated with OS and recurrence rates comparable to those of MC[10,27,28,32]. So, it is clear that not all the patients accepted for LT have a good prognosis and not all the patients discarded for LT based on MC have a dismal one.

In 2009, Mazzaferro *et al*[10] proposed a prognostic model, based on posttransplant estimation of survival probabilities related to the histological stage. This model, known as the "Metroticket", was recently validated by Raj *et al*[49]. In that retrospective analysis of a group of patients with a known 5-year OS of 74%, the model estimated a survival of 70%, statistically not different from the real one. By offering individualized survival predictions, the Metroticket could play a role in the regional organ allocation process. As described, if the expected survival of an individual is similar to that offered to transplanted Milan-in patients, then the LT could be justified depending on the characteristics of each individual region.

However, there are authors who have criticized that both dichotomous and continuous selection models only predict the posttransplant outcome, without taking into account the patient´s survival perspectives without transplantation, geographical differences in terms of donation or waiting list times, or the proportion of patients with and without HCC on the waiting list[50,51].

LDLT for expansion beyond MC
The strategy of LDLT in the context of HCC is different from the LT with deceased donor grafts because of, at least, two reasons. First of all, LDLT does not affect the conventional waiting list, therefore an expansion of the MC could be planned in this context without the fear of affecting other patients waiting for an organ. Second of all, LDLT is a complex procedure that involves not only the recipient, but also a living donor who is a healthy person submitted to a major surgery without a direct benefit. For this reason, the benefit of the recipient should always be evaluated in the context of the risk to the donor, a concept known as "double equipoise"[52].

The majority of LDLT studies regarding CHC expansion criteria have come from Asia, where, for cultural and religious reasons, the cadaveric donation is infrequent (see Tables 1 and 2).

The University of Tokyo published the "5-5 rule criteria" (see Table 1). Using these criteria, 5-year DFS was found to be 94%, while in the patients beyond Tokyo it was only 50%[53]. Two years ago, that same group published the results of their series after a large follow-up. The 5-year recurrence rates were 8% for Milan-out Tokyo-in patients and 6% for Milan-in patients. The OS and DFS rates were comparable between the two groups[54].

The group from Kyoto included dex-gamma-

carboxi prothrobine (DCP) in the criteria for LT (see Table 1)[55]. Applying these criteria, the 5-year OS and recurrence rates were 80% and 7%, respectively, when all the patients (Milan-out Kyoto-in and Milan-in) were considered[56]. The criteria of the University of Kyushu also took into account the DCP, but did not impose a limit on tumor number[57]. By using the Kyushu criteria, the 3- and 5-year DFS was 80%. In a multivariate analysis that considered UCSF, up-to-seven, Tokyo and Kyoto criteria, the Kyushu criteria was the only one statistically related to the DFS.

Another LDLT expanded criteria is the one of Asan Medical Center. The survival and recurrence rates of patients within these criteria were comparable with MC and UCSF survival rates, with the advantage that the Asan criteria can select more patients that can benefit from the transplant[58].

Kim *et al*[59] defined a set of expanded criteria based on reviewing the explant histology of 180 patients, the major portion of this population being submitted to LDLT. The results showed a DFS benefit when the number of tumors was lower than 7, the maximum diameter was smaller than 6 cm, and the AFP was less than or equal to 1000 ng/mL.

Our group also published, this year, the results of a prospective study of 22 patients with BCLC expanded criteria who had submitted to LDLT[14]. The criteria were related to the size and number of the tumors but also to the successful downstaging after locoregional therapies. The results were remarkable, with a 5-year OS of more than 80%. One of the factors that influenced the OS was a "Milan-in" status before the transplant and after performing locoregional therapies as downstaging or bridging therapy (see Table 1). As remarked by other authors, the results of this study seem to favor downstaging over expansion in the context of LDLT, even though the sample size is small[60].

All these studies demonstrate that the expansion of the MC in the context of LDLT does not necessarily associate with worse results. However, the majority of these articles are retrospective analyses of patients selected by the means of explant histological characteristics. Furthermore, some of them analyzed the survival and recurrence rates in Milan-in patients and with expanded criteria all together, which could have biased the results.

The report from the Vancouver Forum on the Care of the Living Donor from 2005 established that LDLT should be performed only if it offers an advantage to the recipient when compared to the alternative of waiting for a deceased donor graft and if the risk of the donor is justified by the expectation of an acceptable outcome of the recipient[61].

One of the main issues in LDLT is the safety of the donor. Clavien's group[62] analyzed the results of several important transplant centers throughout the world and published benchmark values related to acceptable complication rates for donors. That study described acceptable complication rates at discharge values below

26.9% for any complication and 6% for major complications (≥ ⅢA of Clavien-Dindo classification)[63]. Today, the reported donor mortality after LDLT is 0.15%-0.20%[52]. In the particular scenario of LDLT for HCC beyond MC, the concept of double-equipoise should be taken into account, it being unacceptable that a donor should take any risk if the benefit to the recipient is expected to be very low[52]. However, the living donor studies presented above report survival rates comparable to those of LT for MC and lead to optimism regarding the possibility of using LDLT for expanding HCC criteria.

The other important issue related to LDLT for HCC involves the reports of higher rates of recurrence than are related to the conventional LT[23,64]. One possible explanation of these results could be related to the reduced waiting time before LDLT compared with the usual waiting list time for conventional LT. It is possible that this reduced time did not permit drop-out of patients with aggressive HCC[64]. Theoretically, this concept can also apply to the expansion beyond MC. However, a meta-analysis published in 2012 by the group from Guangzhou, China showed no statistical differences between living and cadaveric LT in terms of 5-year OS or recurrence[65]. Of note, in our experience with the application of BCLC expanded criteria for LDLT, the 5-year recurrence rate was approximately 20%, but the OS rate was comparable to that published for Milan-in patients with cadaveric donors[3,4,14].

We believe that as long as the results in terms of survival of selected HCC patients beyond MC (*i.e.* up-to-seven, UCSF, extended criteria BCLC) submitted to LDLT are comparable to those obtained after conventional LT for HCC Milan-in, the utilization of LDLT in this context could be justified.

FUTURE DIRECTIONS

DAA treatment for HCV is one of the most important medical breakthroughs of the last decade. Its impact is already apparent on the United States' liver waiting list, where HCV is no longer the first indication for LT[44]. The liver grafts that are no longer needed for HCV patients could be used to explore the expansion beyond MC. On the other hand, since the association between HCV and HCC is well documented, the DAA treatment is expected to have an impact on the incidence of HCC as well[51]. However, further information is needed in order to explore these scenarios.

Some of the most intriguing future directions of research of HCC treatment are the molecular and genetic analyses and investigations into the relationship of tumor biology and recurrence (see Table 4). It is known today that complex genetic and epigenetic alterations, chromosomal mutations and changes in molecular pathways lead to HCC development[66-71]. Even though these insights have shown much promise in improving HCC treatments, one of their main issues is the retrospective character of the results themselves. In fact, the vast majority of the related studies analyzed

Table 4 Impact of genetic and molecular factors in post-liver transplantation outcome

Ref.	Criteria	Survival, time and rate (%)	Recurrence, time and rate (%)
Schwartz *et al*[68], 2008	FAI < 0.27		5 yr (10)
Dvorchick *et al*[69], 2008	FAI and macrovascular invasion (Pittsburg criteria)	Stage I (FAI ≤ 20% and no macrovascular invasion) - 5 yr DFS (92.8)	
Miltiadous *et al*[67], 2015	Progenitor cell markers (CK19 or S2 signature)	5 yr (67)	5 yr (19)
Sugimachi *et al*[70], 2015	miR-718 and his target gene HOXB8	5 yr (≈ 80)	
Barry *et al*[71], 2012	67 miRNA		
Liese *et al*[72], 2016	miR-214, miR-3187 and MC	5 yr DFS (≈ 90)	

DFS: Disease-free survival; FAI: Fractional allelic imbalance; MC: Milan criteria; miRNA: MicroRNA.

the molecular and genetic characteristics in tumor samples of explanted tissues, which makes any kind of pretransplant selection of these patients based on the tumor biology virtually impossible. However, identification and measurement of genetic markers in serum before LT, like of microRNAs, could be a future direction of investigation[69]. However, more studies are necessary in order to confirm these results.

CONCLUSION

The current medical literature seems to support that modest expansions of HCC LT criteria beyond Milan offer results comparable to those of MC. However, these proposals require further prospective validation using radiological findings collected before LT as a selection tool. As summary, the three important questions cited at the beginning of this article will be addressed in the concluding remarks.

First of all, the effect of possible MC expansion on the waiting list is a variable depending not only on the stage of the HCC patients but also on regional characteristics of the waiting list itself and donors. Thus, we believe that the expansion of MC is a decision that will have to be analyzed carefully in each transplant region and according to the principle of survival benefit for all of the patients on the waiting list.

Second of all, in the Metroticket era, the use of a threshold of acceptable survival, rather than strict dichotomous yes/no criteria, could offer a flexibility to the HCC criteria and may help to expand LT indications beyond the MC in regions where the waiting list pressure permits.

Finally, in the real-life context of cadaveric donor shortage, the use of LDLT is generally accepted. As long as the expansion beyond MC in the context of LDLT offers survival rates comparable to those of accepted indications for LT, its use seems justified.

REFERENCES

1   **World Health Organization IA for R on C.** GLOBOCAN 2012. Liver Cancer. Estimated Incidence, Mortality and Prevalence Worldwide in 2012 [Internet]. [cited 2015-04-05] Accessed April 20, 2018 Available from: URL: www.globocan.iarc.fr/Pages/fact_sheets_cancer.aspx

2   **Mazzaferro V**, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, Ammatuna M, Morabito A, Gennari L. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 1996; **334**: 693-699 [PMID: 8594428 DOI: 10.1056/NEJM199603143341104]

3   **Liver chapter,** 2012 SRTR OPTN Annual Data Report [Internet]. [cited 2015-04-05] Accessed April 20, 2018 Available from: URL: http://srtr.transplant.hrsa.gov/annual_reports/2012/flash/03_liver_13/v2index.html

4   Results - European Liver Transplant Registry - ELTR [Internet]. [cited 2015-04-05] Accessed April 20, 2018 Available from: URL: http://www.eltr.org/-Results-.html

5   **Bruix J**, Sherman M, Practice Guidelines Committee, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma. *Hepatology* 2005; **42**: 1208-1236 [PMID: 16250051 DOI: 10.1002/hep.20933]

6   **Llovet JM**, Di Bisceglie AM, Bruix J, Kramer BS, Lencioni R, Zhu AX, Sherman M, Schwartz M, Lotze M, Talwalkar J, Gores GJ; Panel of Experts in HCC-Design Clinical Trials. Design and endpoints of clinical trials in hepatocellular carcinoma. *J Natl Cancer Inst* 2008; **100**: 698-711 [PMID: 18477802 DOI: 10.1093/jnci/djn134]

7   **Bruix J**, Sherman M, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53**: 1020-1022 [PMID: 21374666 DOI: 10.1002/hep.24199]

8   **European Association For The Study Of The Liver**, European Organisation For Research And Treatment Of Cancer. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. *J Hepatol* 2012; **56**: 908-943 [PMID: 22424438 DOI: 10.1016/j.jhep.2011.12.001]

9   **Yao FY**, Ferrell L, Bass NM, Watson JJ, Bacchetti P, Venook A, Ascher NL, Roberts JP. Liver transplantation for hepatocellular carcinoma: expansion of the tumor size limits does not adversely impact survival. *Hepatology* 2001; **33**: 1394-1403 [PMID: 11391528 DOI: 10.1053/jhep.2001.24563]

10 **Mazzaferro V**, Llovet JM, Miceli R, Bhoori S, Schiavo M, Mariani L, Camerini T, Roayaie S, Schwartz ME, Grazi GL, Adam R, Neuhaus P, Salizzoni M, Bruix J, Forner A, De Carlis L, Cillo U, Burroughs AK, Troisi R, Rossi M, Gerunda GE, Lerut J, Belghiti J, Boin I, Gugenheim J, Rochling F, Van Hoek B, Majno P; Metroticket Investigator Study Group. Predicting survival after liver transplantation in patients with hepatocellular carcinoma beyond the Milan criteria: a retrospective, exploratory analysis. *Lancet Oncol* 2009; **10**: 35-43 [PMID: 19058754 DOI: 10.1016/S1470-2045(08)70284-5]

11 **Roayaie S**, Frischer JS, Emre SH, Fishbein TM, Sheiner PA, Sung M, Miller CM, Schwartz ME. Long-term results with multimodal adjuvant therapy and liver transplantation for the treatment of hepatocellular carcinomas larger than 5 centimeters. *Ann Surg* 2002; **235**: 533-539 [PMID: 11923610 DOI: 10.1097/00000658-20020400 0-00012]

12 **Herrero JI**, Sangro B, Quiroga J, Pardo F, Herraiz M, Cienfuegos JA, Prieto J. Influence of tumor characteristics on the outcome of liver transplantation among patients with liver cirrhosis and

hepatocellular carcinoma. *Liver Transpl* 2001; **7**: 631-636 [PMID: 11460231 DOI: 10.1053/jlts.2001.25458]

13 **Zheng SS**, Xu X, Wu J, Chen J, Wang WL, Zhang M, Liang TB, Wu LM. Liver transplantation for hepatocellular carcinoma: Hangzhou experiences. *Transplantation* 2008; **85**: 1726-1732 [PMID: 18580463 DOI: 10.1097/TP.0b013e31816b67e4]

14 **Llovet JM**, Pavel M, Rimola J, Diaz MA, Colmenero J, Saavedra-Perez D, Fondevila C, Ayuso C, Fuster J, Ginès P, Bruix J, Garcia-Valdecasas JC. Pilot study of living donor liver transplantation for patients with hepatocellular carcinoma exceeding Milan Criteria (Barcelona Clinic Liver Cancer extended criteria). *Liver Transpl* 2018; **24**: 369-379 [PMID: 29140601 DOI: 10.1002/lt.24977]

15 **Duffy JP**, Vardanian A, Benjamin E, Watson M, Farmer DG, Ghobrial RM, Lipshutz G, Yersiz H, Lu DS, Lassman C, Tong MJ, Hiatt JR, Busuttil RW. Liver transplantation criteria for hepatocellular carcinoma should be expanded: a 22-year experience with 467 patients at UCLA. *Ann Surg* 2007; **246**: 502-9; discussion 509-11 [PMID: 17717454 DOI: 10.1097/SLA.0b013e318148c704]

16 **Ecker BL**, Hoteit MA, Forde KA, Hsu CC, Reddy KR, Furth EE, Siegelman ES, Habibollahi P, Ben-Josef E, Porrett PM, Abt PL, Shaked A, Olthoff KM, Levine MH. Patterns of Discordance Between Pretransplant Imaging Stage of Hepatocellular Carcinoma and Posttransplant Pathologic Stage: A Contemporary Appraisal of the Milan Criteria. *Transplantation* 2018; **102**: 648-655 [PMID: 29319629 DOI: 10.1097/TP.0000000000002056]

17 **Llovet JM**, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003; **362**: 1907-1917 [PMID: 14667750 DOI: 10.1016/S0140-6736(03)14964-1]

18 **Llovet JM**, Bruix J, Fuster J, Castells A, Garcia-Valdecasas JC, Grande L, Franca A, Brú C, Navasa M, Ayuso MC, Solé M, Real MI, Vilana R, Rimola A, Visa J, Rodés J. Liver transplantation for small hepatocellular carcinoma: the tumor-node-metastasis classification does not have prognostic power. *Hepatology* 1998; **27**: 1572-1577 [PMID: 9620329 DOI: 10.1002/hep.510270616]

19 **Clavien PA**, Lesurtel M, Bossuyt PM, Gores GJ, Langer B, Perrier A; OLT for HCC Consensus Group. Recommendations for liver transplantation for hepatocellular carcinoma: an international consensus conference report. *Lancet Oncol* 2012; **13**: e11-e22 [PMID: 22047762 DOI: 10.1016/S1470-2045(11)70175-9]

20 **Decaens T**, Roudot-Thoraval F, Hadni-Bresson S, Meyer C, Gugenheim J, Durand F, Bernard PH, Boillot O, Sulpice L, Calmus Y, Hardwigsen J, Ducerf C, Pageaux GP, Dharancy S, Chazouilleres O, Cherqui D, Duvoux C. Impact of UCSF criteria according to pre- and post-OLT tumor features: analysis of 479 patients listed for HCC with a short waiting time. *Liver Transpl* 2006; **12**: 1761-1769 [PMID: 16964590 DOI: 10.1002/lt.20884]

21 **Volk ML**, Vijan S, Marrero JA. A novel model measuring the harm of transplanting hepatocellular carcinoma exceeding Milan criteria. *Am J Transplant* 2008; **8**: 839-846 [PMID: 18318783 DOI: 10.1111/j.1600-6143.2007.02138.x]

22 **Dutkowski P**, De Rougemont O, Müllhaupt B, Clavien PA. Current and future trends in liver transplantation in Europe. *Gastroenterology* 2010; **138**: 802-9.e1-4 [PMID: 20096694 DOI: 10.1053/j.gastro.2010.01.030]

23 **Fisher RA**, Kulik LM, Freise CE, Lok AS, Shearon TH, Brown RS Jr, Ghobrial RM, Fair JH, Olthoff KM, Kam I, Berg CL; A2ALL Study Group. Hepatocellular carcinoma recurrence and death following living and deceased donor liver transplantation. *Am J Transplant* 2007; **7**: 1601-1608 [PMID: 17511683 DOI: 10.1111/j.1600-6143.2007.01802.x]

24 **Mazzaferro V**. Squaring the circle of selection and allocation in liver transplantation for HCC: An adaptive approach. *Hepatology* 2016; **63**: 1707-1717 [PMID: 26703761 DOI: 10.1002/hep.28420]

25 **Colli A**, Fraquelli M, Casazza G, Massironi S, Colucci A, Conte D, Duca P. Accuracy of ultrasonography, spiral CT, magnetic resonance, and alpha-fetoprotein in diagnosing hepatocellular carcinoma: a systematic review. *Am J Gastroenterol* 2006; **101**: 513-523 [PMID: 16542288 DOI: 10.1111/j.1572-0241.2006.00467.x]

26 **Burrel M**, Llovet JM, Ayuso C, Iglesias C, Sala M, Miquel R, Caralt T, Ayuso JR, Solé M, Sanchez M, Brú C, Bruix J; Barcelona

Clínic Liver Cancer Group. MRI angiography is superior to helical CT for detection of HCC prior to liver transplantation: an explant correlation. *Hepatology* 2003; **38**: 1034-1042 [PMID: 14512891 DOI: 10.1053/jhep.2003.50409]

27 **Yao FY**, Xiao L, Bass NM, Kerlan R, Ascher NL, Roberts JP. Liver transplantation for hepatocellular carcinoma: validation of the UCSF-expanded criteria based on preoperative imaging. *Am J Transplant* 2007; **7**: 2587-2596 [PMID: 17868066 DOI: 10.1111/j.1600-6143.2007.01965.x]

28 **Herrero JI**, Sangro B, Pardo F, Quiroga J, Iñarrairaegui M, Rotellar F, Montiel C, Alegre F, Prieto J. Liver transplantation in patients with hepatocellular carcinoma across Milan criteria. *Liver Transpl* 2008; **14**: 272-278 [PMID: 18306328 DOI: 10.1002/lt.21368]

29 **Toso C**, Meeberg G, Hernandez-Alejandro R, Dufour JF, Marotta P, Majno P, Kneteman NM. Total tumor volume and alpha-fetoprotein for selection of transplant candidates with hepatocellular carcinoma: A prospective validation. *Hepatology* 2015; **62**: 158-165 [PMID: 25777590 DOI: 10.1002/hep.27787]

30 **Duvoux C**, Roudot-Thoraval F, Decaens T, Pessione F, Badran H, Piardi T, Francoz C, Compagnon P, Vanlemmens C, Dumortier J, Dharancy S, Gugenheim J, Bernard PH, Adam R, Radenne S, Muscari F, Conti F, Hardwigsen J, Pageaux GP, Chazouillères O, Salame E, Hilleret MN, Lebray P, Abergel A, Debette-Gratien M, Kluger MD, Mallat A, Azoulay D, Cherqui D; Liver Transplantation French Study Group. Liver transplantation for hepatocellular carcinoma: a model including α-fetoprotein improves the performance of Milan criteria. *Gastroenterology* 2012; **143**: 986-94.e3; quiz e14-5 [PMID: 22750200 DOI: 10.1053/j.gastro.2012.05.052]

31 **Sapisochin G**, Goldaracena N, Laurence JM, Dib M, Barbas A, Ghanekar A, Cleary SP, Lilly L, Cattral MS, Marquez M, Selzner M, Renner E, Selzner N, McGilvray ID, Greig PD, Grant DR. The extended Toronto criteria for liver transplantation in patients with hepatocellular carcinoma: A prospective validation study. *Hepatology* 2016; **64**: 2077-2088 [PMID: 27178646 DOI: 10.1002/hep.28643]

32 **Onaca N**, Davis GL, Goldstein RM, Jennings LW, Klintmalm GB. Expanded criteria for liver transplantation in patients with hepatocellular carcinoma: a report from the International Registry of Hepatic Tumors in Liver Transplantation. *Liver Transpl* 2007; **13**: 391-399 [PMID: 17318865 DOI: 10.1002/lt.21095]

33 **Toso C**, Mentha G, Kneteman NM, Majno P. The place of downstaging for hepatocellular carcinoma. *J Hepatol* 2010; **52**: 930-936 [PMID: 20385428 DOI: 10.1016/j.jhep.2009.12.032]

34 **Yao FY**, Ferrell L, Bass NM, Bacchetti P, Ascher NL, Roberts JP. Liver transplantation for hepatocellular carcinoma: comparison of the proposed UCSF criteria with the Milan criteria and the Pittsburgh modified TNM criteria. *Liver Transpl* 2002; **8**: 765-774 [PMID: 12200775 DOI: 10.1053/jlts.2002.34892]

35 **Ravaioli M**, Grazi GL, Piscaglia F, Trevisani F, Cescon M, Ercolani G, Vivarelli M, Golfieri R, D'Errico Grigioni A, Panzini I, Morelli C, Bernardi M, Bolondi L, Pinna AD. Liver transplantation for hepatocellular carcinoma: results of down-staging in patients initially outside the Milan selection criteria. *Am J Transplant* 2008; **8**: 2547-2557 [PMID: 19032223 DOI: 10.1111/j.1600-6143.2008.02409.x]

36 **Bruix J**, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, Christensen E, Pagliaro L, Colombo M, Rodés J; EASL Panel of Experts on HCC. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001; **35**: 421-430 [PMID: 11592607 DOI: 10.1016/S0168-8278(01)00130-1]

37 **Graziadei IW**, Sandmueller H, Waldenberger P, Koenigsrainer A, Nachbaur K, Jaschke W, Margreiter R, Vogel W. Chemoembolization followed by liver transplantation for hepatocellular carcinoma impedes tumor progression while on the waiting list and leads to excellent outcome. *Liver Transpl* 2003; **9**: 557-563 [PMID: 12783395 DOI: 10.1053/jlts.2003.50106]

38 **Heimbach JK**, Kulik LM, Finn RS, Sirlin CB, Abecassis MM, Roberts LR, Zhu AX, Murad MH, Marrero JA. AASLD guidelines

for the treatment of hepatocellular carcinoma. *Hepatology* 2018; **67**: 358-380 [PMID: 28130846 DOI: 10.1002/hep.29086]

39   **Bruix J**, Reig M, Sherman M. Evidence-Based Diagnosis, Staging, and Treatment of Patients With Hepatocellular Carcinoma. *Gastroenterology* 2016; **150**: 835-853 [PMID: 26795574 DOI: 10.1053/j.gastro.2015.12.041]

40   **European Association for the Study of the Liver.** EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol* 2018; **69**: 182-236 [PMID: 29628281 DOI: 10.1016/j.jhep.2018.03.019]

41   **Bruix J**, Fuster J, Llovet JM. Liver transplantation for hepatocellular carcinoma: Foucault pendulum versus evidence-based decision. *Liver Transpl* 2003; **9**: 700-702 [PMID: 12827556 DOI: 10.1053/jlts.2003.50124]

42   **Pavel MC**, Fondevila Campo C, Calatayud Mizrahi D, Ferrer Fabrega J, Sanchez Cabus S, Molina Santos V, Fuster Obregon J, Garcia-Valdecasas Salgado JC. Normothermic perfusion machine in liver transplant with cardiac death donor grafts. *Cir Esp* 2015; **93**: 485-491 [PMID: 26139181 DOI: 10.1016/j.ciresp.2015.05.003]

43   **Adhoute X**, Castellani P, Bourlière M. Impact of direct-acting antiviral agents on the risk for hepatocellular carcinoma. *Transl Gastroenterol Hepatol* 2017; **2**: 110 [PMID: 29354767 DOI: 10.21037/tgh.2017.12.04]

44   **Yang JD**, Larson JJ, Watt KD, Allen AM, Wiesner RH, Gores GJ, Roberts LR, Heimbach JA, Leise MD. Hepatocellular Carcinoma Is the Most Common Indication for Liver Transplantation and Placement on the Waitlist in the United States. *Clin Gastroenterol Hepatol* 2017; **15**: 767-775.e3 [PMID: 28013117 DOI: 10.1016/j.cgh.2016.11.034]

45   **Adam R**, Karam V, Delvart V, O'Grady J, Mirza D, Klempnauer J, Castaing D, Neuhaus P, Jamieson N, Salizzoni M, Pollard S, Lerut J, Paul A, Garcia-Valdecasas JC, Rodríguez FS, Burroughs A; All contributing centers (www.eltr.org); European Liver and Intestine Transplant Association (ELITA). Evolution of indications and results of liver transplantation in Europe. A report from the European Liver Transplant Registry (ELTR). *J Hepatol* 2012; **57**: 675-688 [PMID: 22609307 DOI: 10.1016/j.jhep.2012.04.015]

46   **Jay CL**, Skaro AI, Ladner DP, Wang E, Lyuksemburg V, Chang Y, Xu H, Talakokkla S, Parikh N, Holl JL, Hazen GB, Abecassis MM. Comparative effectiveness of donation after cardiac death versus donation after brain death liver transplantation: Recognizing who can benefit. *Liver Transpl* 2012; **18**: 630-640 [PMID: 22645057 DOI: 10.1002/lt.23418]

47   **Bruix J**, Gores GJ, Mazzaferro V. Hepatocellular carcinoma: clinical frontiers and perspectives. *Gut* 2014; **63**: 844-855 [PMID: 24531850 DOI: 10.1136/gutjnl-2013-306627]

48   **Pomfret EA**, Washburn K, Wald C, Nalesnik MA, Douglas D, Russo M, Roberts J, Reich DJ, Schwartz ME, Mieles L, Lee FT, Florman S, Yao F, Harper A, Edwards E, Freeman R, Lake J. Report of a national conference on liver allocation in patients with hepatocellular carcinoma in the United States. *Liver Transpl* 2010; **16**: 262-278 [PMID: 20209641 DOI: 10.1002/lt.21999]

49   **Raj A**, McCall J, Gane E. Validation of the "Metroticket" predictor in a cohort of patients transplanted for predominantly HBV-related hepatocellular carcinoma. *J Hepatol* 2011; **55**: 1063-1068 [PMID: 21354447 DOI: 10.1016/j.jhep.2011.01.052]

50   **Vitale A**, Volk ML, Gambato M, Zanus G, D'Amico F, Carraro A, Pauletto A, Bonsignore P, Scopelliti M, Polacco M, Russo F, Senzolo M, Burra P, Romano A, Angeli P, Cillo U. Estimation of the harm to the waiting list as a crucial factor in the selection of patients with hepatocellular carcinoma for liver transplantation. *Transplant Proc* 2010; **42**: 1194-1196 [PMID: 20534259 DOI: 10.1016/j.transproceed.2010.03.089]

51   **Forner A**, Reig M, Bruix J. Hepatocellular carcinoma. *Lancet* 2018; **391**: 1301-1314 [PMID: 29307467 DOI: 10.1016/S0140-6736(18)30010-2]

52   **Pomfret EA**, Lodge JP, Villamil FG, Siegler M. Should we use living donor grafts for patients with hepatocellular carcinoma? Ethical considerations. *Liver Transpl* 2011; **17** Suppl 2: S128-S132 [PMID: 21656657 DOI: 10.1002/lt.22356]

53   **Sugawara Y**, Tamura S, Makuuchi M. Living donor liver transplantation for hepatocellular carcinoma: Tokyo University series. *Dig Dis* 2007; **25**: 310-312 [PMID: 17960065 DOI: 10.1159/000106910]

54   **Togashi J**, Akamastu N, Kokudo N. Living donor liver transplantation for hepatocellular carcinoma at the University of Tokyo Hospital. *Hepatobiliary Surg Nutr* 2016; **5**: 399-407 [PMID: 27826554 DOI: 10.21037/hbsn.2016.08.05]

55   **Takada Y**, Ito T, Ueda M, Sakamoto S, Haga H, Maetani Y, Ogawa K, Ogura Y, Oike F, Egawa H, Uemoto S. Living donor liver transplantation for patients with HCC exceeding the Milan criteria: a proposal of expanded criteria. *Dig Dis* 2007; **25**: 299-302 [PMID: 17960063 DOI: 10.1159/000106908]

56   **Kaido T**, Ogawa K, Mori A, Fujimoto Y, Ito T, Tomiyama K, Takada Y, Uemoto S. Usefulness of the Kyoto criteria as expanded selection criteria for liver transplantation for hepatocellular carcinoma. *Surgery* 2013; **154**: 1053-1060 [PMID: 24074704 DOI: 10.1016/j.surg.2013.04.056]

57   **Shirabe K**, Taketomi A, Morita K, Soejima Y, Uchiyama H, Kayashima H, Ninomiya M, Toshima T, Maehara Y. Comparative evaluation of expanded criteria for patients with hepatocellular carcinoma beyond the Milan criteria undergoing living-related donor liver transplantation. *Clin Transplant* 2011; **25**: E491-E498 [PMID: 21518000 DOI: 10.1111/j.1399-0012.2011.01463.x]

58   **Lee SG**, Hwang S, Moon DB, Ahn CS, Kim KH, Sung KB, Ko GY, Park KM, Ha TY, Song GW. Expanded indication criteria of living donor liver transplantation for hepatocellular carcinoma at one large-volume center. *Liver Transpl* 2008; **14**: 935-945 [PMID: 18581465 DOI: 10.1002/lt.21445]

59   **Kim JM**, Kwon CH, Joh JW, Park JB, Lee JH, Kim GS, Kim SJ, Paik SW, Lee SK. Expanded criteria for liver transplantation in patients with hepatocellular carcinoma. *Transplant Proc* 2014; **46**: 726-729 [PMID: 24767334 DOI: 10.1016/j.transproceed.2013.11.037]

60   **Mehta N**, Yao FY. Living donor liver transplantation for hepatocellular carcinoma: To expand (beyond Milan) or downstage (to Milan)? *Liver Transpl* 2018; **24**: 327-329 [PMID: 29351366 DOI: 10.1002/lt.25017]

61   **Barr ML**, Belghiti J, Villamil FG, Pomfret EA, Sutherland DS, Gruessner RW, Langnas AN, Delmonico FL. A report of the Vancouver Forum on the care of the live organ donor: lung, liver, pancreas, and intestine data and medical guidelines. *Transplantation* 2006; **81**: 1373-1385 [PMID: 16732172 DOI: 10.1097/01.tp.0000216825.56841.cd]

62   **Rössler F**, Sapisochin G, Song G, Lin YH, Simpson MA, Hasegawa K, Laurenzi A, Sánchez Cabús S, Nunez MI, Gatti A, Beltrame MC, Slankamenac K, Greig PD, Lee SG, Chen CL, Grant DR, Pomfret EA, Kokudo N, Cherqui D, Olthoff KM, Shaked A, García-Valdecasas JC, Lerut J, Troisi RI, De Santibanes M, Petrowsky H, Puhan MA, Clavien PA. Defining Benchmarks for Major Liver Surgery: A multicenter Analysis of 5202 Living Liver Donors. *Ann Surg* 2016; **264**: 492-500 [PMID: 27433909 DOI: 10.1097/SLA.0000000000001849]

63   **Dindo D**, Demartines N, Clavien PA. Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey. *Ann Surg* 2004; **240**: 205-213 [PMID: 15273542 DOI: 10.1097/01.sla.0000133083.54934.ae]

64   **Kulik L**, Abecassis M. Living donor liver transplantation for hepatocellular carcinoma. *Gastroenterology* 2004; **127**: S277-S282 [PMID: 15508095 DOI: 10.1053/j.gastro.2004.09.042]

65   **Liang W**, Wu L, Ling X, Schroder PM, Ju W, Wang D, Shang Y, Kong Y, Guo Z, He X. Living donor liver transplantation versus deceased donor liver transplantation for hepatocellular carcinoma: a meta-analysis. *Liver Transpl* 2012; **18**: 1226-1236 [PMID: 22685095 DOI: 10.1002/lt.23490]

66   **Miltiadous O**, Sia D, Hoshida Y, Fiel MI, Harrington AN, Thung SN, Tan PS, Dong H, Revill K, Chang CY, Roayaie S, Byrne TJ, Mazzaferro V, Rakela J, Florman S, Schwartz M, Llovet JM. Progenitor cell markers predict outcome of patients with hepatocellular carcinoma beyond Milan criteria undergoing liver

transplantation. *J Hepatol* 2015; **63**: 1368-1377 [PMID: 26220754 DOI: 10.1016/j.jhep.2015.07.025]

67   **Schwartz M**, Dvorchik I, Roayaie S, Fiel MI, Finkelstein S, Marsh JW, Martignetti JA, Llovet JM. Liver transplantation for hepatocellular carcinoma: extension of indications based on molecular markers. *J Hepatol* 2008; **49**: 581-588 [PMID: 18602719 DOI: 10.1016/j.jhep.2008.03.032]

68   **Dvorchik I**, Schwartz M, Fiel MI, Finkelstein SD, Marsh JW. Fractional allelic imbalance could allow for the development of an equitable transplant selection policy for patients with hepatocellular carcinoma. *Liver Transpl* 2008; **14**: 443-450 [PMID: 18266211 DOI: 10.1002/lt.21393]

69   **Sugimachi K**, Matsumura T, Hirata H, Uchi R, Ueda M, Ueo H, Shinden Y, Iguchi T, Eguchi H, Shirabe K, Ochiya T, Maehara Y, Mimori K. Identification of a bona fide microRNA biomarker in serum exosomes that predicts hepatocellular carcinoma recurrence after liver transplantation. *Br J Cancer* 2015; **112**: 532-538 [PMID: 25584485 DOI: 10.1038/bjc.2014.621]

70   **Barry CT**, D'Souza M, McCall M, Safadjou S, Ryan C, Kashyap R, Marroquin C, Orloff M, Almudevar A, Godfrey TE. Micro

RNA expression profiles as adjunctive data to assess the risk of hepatocellular carcinoma recurrence after liver transplantation. *Am J Transplant* 2012; **12**: 428-437 [PMID: 22008552 DOI: 10.1111/j.1600-6143.2011.03788.x]

71   **Liese J**, Peveling-Oberhag J, Doering C, Schnitzbauer AA, Herrmann E, Zangos S, Hansmann ML, Moench C, Welker MW, Zeuzem S, Bechstein WO, Ulrich F. A possible role of microRNAs as predictive markers for the recurrence of hepatocellular carcinoma after liver transplantation. *Transpl Int* 2016; **29**: 369-380 [PMID: 26697811 DOI: 10.1111/tri.12733]

72   **Yao FY**, Mehta N, Flemming J, Dodge J, Hameed B, Fix O, Hirose R, Fidelman N, Kerlan RK Jr, Roberts JP. Downstaging of hepatocellular cancer before liver transplant: long-term outcome compared to tumors within Milan criteria. *Hepatology* 2015; **61**: 1968-1977 [PMID: 25689978 DOI: 10.1002/hep.27752]

73   **Millonig G**, Graziadei IW, Freund MC, Jaschke W, Stadlmann S, Ladurner R, Margreiter R, Vogel W. Response to preoperative chemoembolization correlates with outcome after liver transplantation in patients with hepatocellular carcinoma. *Liver Transpl* 2007; **13**: 272-279 [PMID: 17256758 DOI: 10.1002/lt.21033]

Diagnosis and management of fibromuscular dysplasia and segmental arterial mediolysis in gastroenterology field: A mini-review

Masayoshi Ko, Kenya Kamimura, Kohei Ogawa, Kentaro Tominaga, Akira Sakamaki, Hiroteru Kamimura, Satoshi Abe, Kenichi Mizuno, Shuji Terai

Masayoshi Ko, Kenya Kamimura, Kohei Ogawa, Kentaro Tominaga, Akira Sakamaki, Hiroteru Kamimura, Satoshi Abe, Kenichi Mizuno, Shuji Terai, Division of Gastroenterology and Hepatology, Graduate School of Medical and Dental Sciences, Niigata University, Niigata 9518510, Japan

ORCID number: Masayoshi Ko (0000-0002-0792-0868); Kenya Kamimura (0000-0001-7182-4400); Kohei Ogawa (0000-0001-6681-4427); Kentaro Tominaga (0000-0001-6792-1005); Akira Sakamaki (0000-0002-9368-7272); Hiroteru Kamimura (0000-0002-9135-3092); Satoshi Abe (0000-0003-1153-0720); Kenichi Mizuno (0000-0003-4702-9874); Shuji Terai (0000-0002-5439-635X).

Abstract

The vascular diseases including aneurysm, occlusion, and thromboses in the mesenteric lesions could cause severe symptoms and appropriate diagnosis and treatment are essential for managing patients. With the development and improvement of imaging modalities, diagnostic frequency of these vascular diseases in abdominal lesions is increasing even with the small changes in the vasculatures. Among various vascular diseases, fibromuscular dysplasia (FMD) and segmental arterial mediolysis (SAM) are noninflammatory, nonatherosclerotic arterial diseases which need to be diagnosed urgently because these diseases could affect various organs and be lethal if the appropriate management is not provided. However, because FMD and SAM are rare, the cause, prevalence, clinical characteristics including the symptoms, findings in the imaging studies, pathological findings, management, and prognoses have not been systematically summarized. Therefore, there have been neither standard diagnostic criteria nor therapeutic methodologies established, to date. To systematically summarize the information and to compare these disease entities, we have summarized the characteristics of FMD and SAM in the gastroenterological regions by reviewing the cases reported thus far. The information summarized will be helpful for physicians treating these patients in an emergency care unit and for the differential diagnosis of other diseases showing severe abdominal pain.

Core tip: The vascular diseases in the abdominal lesions needs to be appropriately diagnosed and treated as it could be lethal if the appropriate management is not provided. Mesenteric ischemia caused by the atherosclerotic changes is rather famous however, fibromuscular dysplasia (FMD) and segmental arterial mediolysis (SAM) which are noninflammatory, nonatherosclerotic arterial diseases are rare and the cause, prevalence, clinical characteristics including the symptoms, findings in the imaging studies, pathological findings, management, and prognoses have not been systematically summarized. Therefore, we have summarized the characteristics of FMD and SAM in the gastroenterological regions and review the cases reported thus far. The information summarized will be helpful for physicians treating these patients in an emergency care unit and for the differential diagnosis of other diseases showing severe abdominal pain.

INTRODUCTION

A literature search was conducted using PubMed and Ovid, with the term "fibromuscular dysplasia" or "segmental arterial mediolysis" and "mesenteric" to extract studies published in the last 55 years for fibromuscular dysplasia and in the last 21 years for segmental arterial mediolysis. We summarized the available information on demographics, clinical symptoms, image studies, histological findings, treatment, and clinical course.

FIBROMUSCULAR DYSPLASIA

Clinical characteristics

The detailed clinical and pathological classification of fibromuscular dysplasia (FMD) was first reported by Harrison and McCormack in 1971[1]. Since then, several studies regarding clinical course and histological data have been published, and recently, data from the first 447 patients from the United States Registry (US Registry) for FMD have been reported[1]. FMD is a noninflammatory, nonatherosclerotic arterial disease of the medium-sized arteries throughout the body, which could lead to arterial stenosis, occlusion, aneurysm, and dissection[2].

The details of the disease have not yet been clarified; however, it is typically found in the renal, extracranial, carotid, and vertebral arteries[2].

The disease is rare, with a frequency of 0.02%, predominantly occurring in women (91%) with a mean age of 55.7 ± 13.1 years especially in the Caucasian (95.4%)[2].

The mean patient age at first symptom or sign of FMD was 47.2 years (range, 5-83 years)[2]. The mechanisms underlying the pathogenesis of FMD are still poorly understood; however, smoking, hormones, HLA-DRw6 polymorphism, and physiological stimulation have been reported to be risk factors[3]. For example, a significant dose-response relationship between cigarette smoking and the presence of FMD has been reported, with an odds ratio of 8.6 when having smoked more than 10 pack-years of cigarettes[3]. The risk of HLA-DRw6 was reported with an odds ratio of 5.0, adjusted for the level of smoking[3].

FMD can occur in any medium-sized arteries throughout the body, and dissection and aneurysm have been identified in 19.7% and 17.0% of FMD patients, respectively. The three major sites affected with dissection are the carotid arteries (14.8% of all patients enrolled), followed by renal arteries (4.3%), and vertebral arteries (3.4%)[2,4]. The three major sites affected with aneurysm are the renal arteries (5.6% of all patients enrolled), followed by carotid arteries (3.6%), and the aorta (3.4%)[2]. FMD in abdominal lesions, classified as mesenteric FMD, which is caused by the celiac and mesenteric arteries, is a rare condition and often presents as an incidental diagnosis[2]. On the basis of the US Registry data, mesenteric ischemia was reported in only 1.3% of cases, with aneurysm and dissection in these vessels accounting for 6.8% and 22.3% of all cases reported, respectively[2].

Symptoms and imaging

The clinical symptoms depend on the vessels involved. When the renal arteries are affected, renovascular hypertension can be observed. Thus, when the carotid arteries are affected, headache, pulsatile tinnitus, and dizziness are the major symptoms[2]. Mesenteric FMD involves the celiac and mesenteric arteries; therefore, mesenteric ischemic symptoms occur, including unspecific abdominal pain. We reviewed the literature describing the cases and have presented the information in Table 1[5-37]. Our literature review summarized a total of 39 cases of mesenteric FMD, showing predominance in women, as reported, and the median age was 45.2 years (range: 19-78 years). Regarding the risk factors, four patients smoked (10%), two patients had smoking histories (5%), and one patient had taken oral contraceptive pills (2.6%). The most common presenting symptom was abdominal pain (62%), followed by hypertension, diarrhea, nausea or vomiting, and headache. Although approximately 80% of cases showed symptom improvement, eight patients (20%) died because of the severity of the intestinal

Table 1 Summary of mesenteric fibromuscular dysplasia reported to date

Case (n)	Ref.	Age (yr)	Gender (Male/Female)	Risk factors	Symptoms	Vessels Involved	CT	Angiography	Pathology	Treatment	Anti-hypertensive drug	Anti-coagulants	Outcome
1	[5]	62	M	N/A	Upper abdominal pain, hemoperitoneum, shock	Celiac, SMA, IMA, RA	N/A	N/A	Intimal thickening in the branches of the SMA and IMA.	Laparotomy	None	None	Died
2	[6]	45	F	N/A	Abdominal pain	SMA, RA	N/A	N/A	N/A	Ileal resection	N/A	N/A	Improved
3	[6]	50	F	N/A	Hypertension, abdominal pain, diarrhea	SMA, RA, iliac	N/A	Stenosis and string-of-beads like Appearance in the SMA	N/A	SMA revascularization	N/A	N/A	Improved
4	[7]	73	F	N/A	N/A	Celiac, SMA, iliac	N/A	N/A	N/A	N/A	N/A	N/A	Improved
5	[7]	42	F	N/A	N/A	SMA, RA	N/A	N/A	N/A	N/A	N/A	N/A	Improved
6	[7]	50	F	N/A	Hypertension	Celiac, SMA, RAl	N/A	Minimal defects in the SMA and RA; stenosis of celiac artery	N/A	N/A	N/A	N/A	Improved
7	[7]	37	F	N/A	Visceral ischemic symptoms	Celiac, SMA, RA	N/A	N/A	N/A	Revascularization	N/A	N/A	Improved
8	[7]	47	F	N/A	Hypertension, abdominal pain	Celiac, SMA, RA	N/A	Defects in the SMA and RA	Medial hyperplasia	None	N/A	N/A	Died
9	[8]	41	F	N/A	Hypertension	SMA, internal carotid, RA, iliac	N/A	Corkscrew and string-of-beads like appearance in the RA, carotid, iliac artery	Replacement of the normal media with disorganized fibrous and muscular hyperplasia	Thromboendarerectomy on SMA	N/A	N/A	Died
10	[9]	64	F	N/A	Unconsciousness	SMA, circle of Willis	N/A	N/A	Medial hyperplasia	None	None	None	Died
11	[10]	21	M	N/A	Hypertension	Celiac, SMA, RA, carotid	N/A	Stenosis of celiac, SMA, RA	Intimal fibroplasia	Anti-hypertensive drug; revascularization of carotid artery; angioplasty of RA	Yes, N/A	N/A	Improved
12	[10]	20	F	N/A	Hypertension	SMA, IMA, RA, carotid	N/A	Stenosis of carotid, renal, SMA. Total occlusion of the IMA.	Intimal fibroplasia	Anti-hypertensive drug; subclavian-carotid bypass; vascular reconstruction of the kidney	Yes, N/A	N/A	Improved
13	[11]	55	M	N/A	N/A	SMA	N/A	N/A	Intimal hyperplasia in SMA	None	N/A	N/A	N/A
14	[12]	44	F	N/A	Asymptomatic bruit of the aortoiliac system	Celiac, SMA, RA, iliac	N/A	String-of-beads like appearance of iliac artery; aneurysms of SMA, RA	Intimal fibrosis with development of fibrosis	Resection and reconstruction	N/A	N/A	Improved
15	[13]	58	F	N/A	Body weight loss	Celiac, SMA, IMA, RA, iliac, aorta	N/A	Occlusion of celiac, SMA, IMA.	N/A	Open surgery	N/A	N/A	Improved
16	[14]	46	F	None (non-smoker)	Palpitations, headache, hypertension	Celiac, SMA	N/A	Aneurysms in the right RA, celiac; occlusion in the left gastric artery	Muscle hypertrophy and disorganisation of elastic tissue of the media in celiac artery.	Surgical ligation	N/A	N/A	Improved

No	Ref	Age	Sex	Smoking	Symptoms	Arteries	Imaging finding	Angiographic finding	Pathology	Treatment	Medication		Outcome
17	[15]	60	M	None (non-smoker)	Left abdominal pain, diarrhea	SMA	Irregular nodular thickening in transverse colon.	Stenoses of the SMA	Intimal fibrosis and focal replacement of medial smooth-muscle fibers by fibrous tissue	Splenic flexure resection and angioplasty	N/A	N/A	Improved
18	[16]	54	F	Smoking	Hypertension, headache, abdominal pain	SMA, RA, coronary	Liver cyst	Stenosis of the coronary arteries	Intimal hyperplasia in SMA, RA, coronary, splenic, intrahepatic artery	Anti-hypertensive drug	α,β-blocker → Ca blocker	N/A	Died
19	[17]	39	M	N/A	Melena, lower abdominal pain	Jejunal, Sigmoid	N/A	String-of-beads like appearance in the jejunal and sigmoid arteries.	Adventitia is thickened by fibroplasia	Resection of the jejunum	N/A	N/A	Improved
20	[18]	23	M	N/A	Hypertension	Celiac, SMA, RA, carotid, vertebral, opthalmic, superficial temporal, iliac, lumber, intercostal	Hematomasin the paraduodenal and right superior gluteral lesion and splenic infarction	Multiple saccular aneurysms in the celiac, SMA, RA< splenic, hepatic, iliac, lumber, and intercostal arteries	Mediolytic FMD with segmental dissection and thrombosis	Embolization of the gastroduodenal and right SMA to prevent hemorrhage	N/A	N/A	Improved
21	[19]	33	M	N/A	Abdominal pain	SMA	N/A	String-of-beads like appearance in the SMA	Thickening of the media due to hyperplasia in SMA	Ileal resection	N/A	N/A	Improved
22	[20]	78	F	N/A	Hypertension, abdominal pain, hemoperitoneum.	SMA, RA, colonary	Dilated loop of the small bowel and fluid in the peritoneal cavity.	N/A	Medial and perimedial fibrodysplasia, forms the characteristic petal-like appearance in SMA.	None	None	None	Died
23	[21]	43	M	None (non-smoker)	No symptoms	SMA, iliac	SMA aneurysm	Aneurysms in the SMA, hepatic artery, splenic artery, jejunal artery, iliac arteries.	Medial fibrodysplasia in the arterial walls	Aneurysm resection and arterial reconstruction	N/A	N/A	Improved
24	[22]	48	F	None (non-smoker)	Abdominal pain, hemoperitoneum	Celiac, SMA, RA	N/A	Multiple small aneurysms in celiac, SMA, RA	N/A	Surgical hemostasis and anti-hypertensive drugs	β-blocker	N/A	Improved
25	[23]	57	F	Smoking (40 packs/yr)	Abdominal pain, weight loss, anorexia, nausea, vomiting, diarrhea.	Celiac, SMA	Nothing particular	Stenosis of the celiac artery and SMA	Medial thickening, smooth muscle hyperplasia in SMA and celiac artery	Aortoiliac and aorto-SMA bypass	N/A	N/A	Died
26	[24]	48	F	Smoking (20 packs/yr)	Abdominal pain	Celiac, SMA, IMA	N/A	Occlusion of the celiac, SMA; enlarged hypertrophic IMA	Intimal fibroplasia and an increased deposition of fibrous tissue in the vessel wall media	Reimplantation of the SMA	N/A	N/A	Improved
27	[25]	38	M	Smoking	Gastrointestinal bleeding, anemia	SMA, IMA	N/A	Ectasia in IMA; string-of-beads like appearance in the SMA	Thickening and hyalinization of medium sized vessel walls, with intimal proliferation.	Ileal resection	N/A	N/A	Improved

No.	Ref	Age	Sex	Smoking history	Symptoms	Arteries	Finding	Imaging finding	Pathology	Treatment	Med 1	Med 2	Outcome
28	[26]	Not provided	Not provided	None (non-smoker)	Abdominal pain, distension, constipation	SMA	N/A	N/A	Thick cuff (petal like) of smooth muscle proliferation with normal intima and media in mesenteric artery	Right hemicolectomy	N/A	N/A	Improved
29	[27]	43	F	Smoking (10 cigarettes daily for 20 yr)	Hypertension, headache	SMA, RA	N/A	String-of-beads like appearance in the right RA and SMA; stenosis and multiple irregularities in the left RA	N/A	Angioplasty and anti-hypertensive drugs	Yes, N/A	N/A	Improved
30	[28]	38	M	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Improved
31	[29]	43	F	N/A	Hypertension, abdominal pain, headache	SMA, RA	Aneurysms in the left RA	Aneurysms in the left RA with sever fibrodysplastic stenosis; string-of-beads like appearance in the right RA; stenosis in SMA	Intimal fibroplasia, lost of internal elastic lamina, and massive destruction of the media in the aneurysm walls	Aneurysm resection and aortorenal bypass and percutaneous transluminal angioplasty	N/A	N/A	Improved
32	[30]	44	F	Oral contraceptive pills	Hypertension, abdominal pain, diarrhea, vomiting	SMA	Stenosis of SMA and nonspecific colitis	stenosis in SMA	N/A	Angioplasty	N/A	N/A	Improved
33	[31]	30	M	N/A	Abdominal pain, hypertension	Celiac, SMA, RA, iliac	Dissections of the celiac, SMA, left RA, and external iliac artery	stenosis in the right RA	N/A	Anti-platelet and anti-hypertensive therapy and angioplasty for right renal artery.	β-blocker, Ca blocker	warfarin, aspirin (100mg)	Improved
34	[32]	47	F	N/A	Abdominal pain, diarrhea, hypertension	All abdominal arteries	A partial occlusion of the celiac artery and a total occlusion of the SMA	N/A	Intimal and medial proliferation	Anti-hypertensive drug	Yes, N/A	N/A	Died
35	[33]	47	F	None (non-smoker)	Nausea, early satiety, abdominal pain	Celiac, SMA	N/A	Stenosis of the SMA, hypertrophy of the gastroduodenal artery and pancreaticoduodenal arteries	N/A	An aorto-superior mesenteric artery and an aorto-hepatic artery bypass.	N/A	N/A	Improved
36	[34]	19	F	N/A	Abdominal pain, vomiting	SMA, RA	N/A	Stenosis of the origin of the SMA and multiple aneurysms involving the proximal SMA. Right renal artery is mild irregularity.	N/A	Resection of the aneurysmal segment in the SMA; aorto-SMA interposition graft with polytetrafluoroethylene	N/A	N/A	Improved
37	[35]	52	M	Smoking	Abdominal pain	IMA	Stenosis of the IMA	N/A	Necrosis of the mucosa; fibrosis of the intima	Left hemicolectomy	N/A	N/A	Improved

38	[36]	20	F	N/A	Abdominal pain, hemorrhagic shock	SMA, right gastroepiploic, jejunal	Intraperitoneal bleeding in the omental bursa and mesentery of the transverse colon	String-of-beads like appearance in the jejunal artery	N/A	Transcatheter arterial embolization	N/A	N/A	Improved
39	[37]	61	F	N/A	Abdominal pain	SMA, IMA, RA	N/A	Multiple aneurysms and stenoses in SMA, IMA, RA	Multiple tears and dissections of the medial layer and fibrointimal thickening	Anti-coagulation	None	Yes, N/A	Improved

M: Male; F: Female; N/A: Data not applicable; SMA: Superior mesenteric artery; IMA: Inferior mesenteric artery; RA: Renal artery; CT: Computed tomography.

ischemia.

Reflecting the changes in stenosis, dissection, and aneurysm in the medium-sized arteries, FMD leads to the narrowing of the vasculature and shows a beaded appearance[38]. Therefore, catheter-based angiography has been considered to be the gold standard imaging modality; however, recent progress in imaging, such as computed tomography (CT) with high resolution, could support the diagnoses by determining the vessels affected by the disease. With the information obtained from imaging, the disease is classified into four types: multifocal, which comprises 62% of cases, showing multiple stenosis and string-of-beads; tubular, which comprises 14% of cases, showing long concentric stenosis; focal, which comprises 7% of cases, showing short stenosis of less than 1 cm; and mixed[39]. Our summary also showed aneurysms, stenosis, dissection, and occlusions in the cases for which information was available.

Histology

Histopathological findings are characteristics of the disease; thinned media and thickened fibromuscular ridges in which the arterial muscle is replaced by the fibroplasia can be observed. Based on this, the characteristic classification of FMD is essentially based on the arterial layer in which the dysplasia is predominant: intimal fibroplasia, medial fibroplasia, perimedial fibroplasia, medial hyperplasia, and adventitial fibroplasia[40-43]. Intimal fibroplasia, a relatively rare form of the disease, is characterized by focal eccentric or circumferential protuberant intimal proliferation. Medial fibroplasia, the most common type, accounts for more than 70% of this disorder, and angiography shows a typical "string of beads" appearance. Perimedial fibroplasia is the second-most common form of this disorder and is characterized by the accumulation of circumferential aggregations of elastic tissue between the media and the adventitia. Medial hyperplasia is an uncommon form and is characterized by apparent hyperplasia of normal medial smooth muscle with minimal architectural disorganization. Adventitial fibroplasia is characterized by collagenous fibroplasia encircling the adventitia and extending into the surrounding periarterial fibroadipose tissue[20]. In our case summary, fibromuscular change was confirmed histologically in the medial layer in 10 patients (26%), the intimal layer in 9 patients (23%), in both layers in 5 patients (13%), and in the adventitia layer in 1 patient (2.6%) (Table 1).

Treatment

The long-term outcomes of this disease entity have not been clarified to date, and no randomized clinical trials have been conducted to develop a standard treatment for this disease. Therapeutic options have been chosen on the basis of factors such as disease location, symptoms, prior history of symptoms, and the presence and size of aneurysms. Given that FMD often shows ischemic changes causing hypertension and stroke, most patients are treated with anti-platelet, anti-thrombotic, and anti-hypertensive therapy[44]. Anti-hypertensive medications are administered to 71.7% of patients. The median number of medications patients received was one, and 21.5% of patients received three or more anti-hypertensive medications. The most commonly used agents were beta-blockers (40.0%), diuretics (31.3%), and calcium channel antagonists (25.7%). A total of 21.0% of patients received an angiotensin-converting enzyme inhibitor, 21.6% received an angiotensin receptor blocking agent, and 0.8% received both[44]. The use of anti-hypertensive agents is related to the history of hypertension medication, body mass index, and renal function[44]. The use of anti-platelet treatment is associated with cerebrovascular involvement[44]; however, for this entity of medicines, further studies are necessary to determine the clinically meaningful patient outcomes. In our case

summary, insufficient information about medications was provided; thus, the actual number treated with antihypertensive therapy might be lower than 18% (Table 1). Anti-coagulation therapy was attempted for two patients (5%), including one patient each receiving warfarin and aspirin.

Vascular intervention and surgery for revascularization are considered with the appropriate clinical symptoms and are rarely performed other than for the renal artery[44]. For the renal artery, endovascular revascularization using the percutaneous transluminal angioplasty technique or surgical procedures are considered when the patients show hypertension resistant to a regimen of three anti-hypertension drugs, including diuretics, or in cases of renal artery aneurysm or renal artery dissection[2,4,38]. Thus far, no randomized clinical trials of revascularization *vs* medication have been conducted. For other arteries, including the carotid artery, given that FMD is not an atherosclerotic disease, stenting or surgical procedures are not the standard therapy, and medication with anti-platelet, anti-coagulant, and anti-hypertensive agents are the main treatment. However, when symptomatic, interventional radiology using the percutaneous transluminal angioplasty technique can be considered, although it is controversial[38]. In our case summary, open surgery was performed on 23 patients (59%) and endovascular intervention was performed on 9 patients (23%).

Prognosis

Though the prognosis is basically good, when FMD affects the cerebrovascular system, there is a risk of cerebral infarction and rupture. A larger number of cases are necessary to accumulate the information useful to conduct randomized clinical trials.

SEGMENTAL ARTERIAL MEDIOLYSIS

Clinical characteristics

Segmental arterial mediolysis (SAM) was first reported by Slavin and Gonzalez-Vitale in 1976[45] and is a rare disease entity for which 50 cases have been reported to date. It is defined as a nonatherosclerotic, noninflammatory disruption of the arterial medial layer of a medium- to large-sized artery. Histologically, it is characterized as vacuolization and lysis of the outer arterial media[45]. Because of its rarity and difficulty in differential diagnosis from the other vascular diseases, clinical information is insufficient, and little is known to date; however, no significant predominance of sex or age has been reported. The mechanisms underlying the pathogenesis of SAM that have been reported as risk factors are hypoxia, shock, aging, hypertension, circulatory disturbance, arteriospasm, and other vasoconstrictor stimuli[45-47].

Symptoms and imaging

For the abdominal lesion, the most common symptom is nonspecific abdominal and flank pain[46]; diarrhea, nausea, back pain, headache, hypertension, loss of consciousness, and hemiparesis have also been known to be symptoms, although not specific[47]. We reviewed the literature describing the cases and have summarized the information in Table 2[47-71]. The studies reported a total of 26 cases of mesenteric SAM, of which 17 were men and 9 were women, with a slight predominance in men. The median age was 53 years (range: 25-79 years). The most common presenting symptom was abdominal pain (78%), followed by various symptoms, including shock, diarrhea, nausea, back pain, headache, anorexia, hypertension, hemiparesis, and loss of consciousness (Table 2).

With the development of various imaging modalities, it has been reported that, in various combinations, SAM typically affects splenic, celiac, hepatic, mesenteric, and renal arteries in the abdominal lesion[47,72]. Because of the involvement of the celiac artery, splenic arterial aneurysm is frequently found, and its rupture could affect the prognosis. Angiography reveals aneurysms, dissections, occlusions, and stenosis; however, the findings could overlap with those found in collagen vascular diseases and FMD. Therefore, the differential diagnoses between the vascular diseases are based on the histopathological findings. SAM is difficult to distinguish from FMD, although FMD shows predominance in young women and affects renal arteries causing hypertension, whereas SAM commonly affects the celiac arteries. In addition, the clinical course shows ischemic changes in FMD, whereas SAM often causes profuse bleeding from the intestinal arteries. However, these findings often overlap each other; therefore, accumulation of more detailed information is necessary.

Histology

Although the suspicion of SAM is the basis of clinical and radiological features, the gold standard for diagnosis is a pathological finding involving injurious and reparative phases in the arterial lesions of the surgical specimens. These injurious states include mediolysis, separation of the outer media, and formation of arterial gaps; key is that there is no evidence of inflammation. These changes reflect the vascular aneurysms frequently found as angiographic features of this condition. Commonly, the inflammatory markers are negative and genetic diagnosis for collagen vascular disorders shows a normal pattern.

Treatment

The long-term prognosis is unclear, and no standard therapeutic strategy has been proposed, to date; however, given that some SAM cases showed sudden the onset of aneurysm rupture, the condition could be life threatening. Therefore, SAM treatment includes embolization, bypass, and resection of the injured arteries. In addition, anti-hypertensive therapy[28] could prevent further worsening of the arterial lesions. Anti-

Table 2 Summary of mesenteric segmental arterial mediolysis reported to date

Case (n)	Ref.	Age (yr)	Gender (Male/Female)	Risk factors	Symptoms	Vessels Involved	CT	Angiography	Pathology	Treatment	Anti-hypertensive drug	Anti-coagulants	Outcome
1	[48]	65	F	N/A	Abdominal pain	SMA	N/A	Beaded appearance and stenosis of the MCA	Lysis and destruction in the media and intima	Resection of aneurysm in MCA	N/A	N/A	Improved
2	[49]	56	F	N/A	Abdominal pain	IMA	Intraabdominal hemorrhage	Aneurysm in IMA	N/A	Left hemicolectomy	N/A	N/A	Improved
3	[50]	78	M	N/A	Abdominal pain, diarrhea, shock	SMA	N/A	N/A	Destruction of the tunica interna and media in MCA	Emergent surgery (right hemicolectomy); a large hematoma and a ruptured aneurysm upon the surgery	N/A	N/A	Improved
4	[51]	35	F	N/A	Abdominal pain, perforation on transverse colon	SMA	Occlusion of the mesenteric vein and ischemic colitis	Unremarkable	Segmental vacuolar degeneration of smooth muscle with areas of wall thinning	Resection of terminal ileum	N/A	N/A	Died
5	[52]	52	M	N/A	Sudden hemiparesis, hypertension	Celiac, SMA, IMA hepatic artery	Aneurysm in the celiac, hepatic, SMA	Aneurysms in celiac, SMA, ICA, hepatic; stenoses in celiac and SMA	Multiple segmental mediolysis lesions of the muscular and elastic fibers of the media	Reconstruction of hepatic and celiac artery using autologous saphenous vein graft	N/A	N/A	Improved
6	[53]	49	M	N/A	Abdominal pain, shock	SMA	Large hematoma surrounding a high-density aneurysm	Beaded appearance in SMA	Multifocal fragmentation of the elastic fibers of the media	Right hemicolectomy	N/A	N/A	Improved
7	[54]	57	M	N/A	Abdominal pain	SMA, hepatic	Small aneurysm at the middle colic artery and mesenteric hematoma	Aneurysm and stenosis of the celiac, SMA, hepatic artery	N/A	Embolization with N-butyl cyanoacrylate for aneurysm in the SMA	N/A	N/A	Improved
8	[54]	76	F	N/A	Abdominal pain, nausea	IMA	Mesenteric hematoma	Aneurysm in IMA	N/A	Embolization with coil	N/A	N/A	Died
9	[55]	59	M	N/A	Abdominal pain, shock	SMA, RA, gastroepiploic, splenic	SMA dissection, aneurysm in RA, gastroepiploic, splenic artery; rupture of the splenic aneurysm	Saccular aneurysms and multiple stenotic region in gastroepiploic artery	Medial island spared from mediolysis	Emergency embolization of the splenic artery, resection of aneurysm in the gastroepiploic	N/A	N/A	Improved
10	[56]	57	M	N/A	Abdominal pain, diarrhea	SMA	Ascites throughout the abdomen	Aneurysm in SMA	N/A	Transcatheter arterial embolization	N/A	N/A	Improved
11	[57]	60	M	N/A	N/A	SMA	Rupture of the aneurysm of the MCA	Multiple beaded patters and aneurysm in SMA	N/A	Surgical resection	N/A	N/A	Improved
12	[47]	25	F	N/A	Anorexia, abdominal pain, diarrhea	SMA, hepatic	Ischemic colitis of the splenic flexure	Occlusion of IMA; stenoses of the hepatic artery	Patchy, isolated destruction of the arterial media involving both the internal and external elastic laminae	Partial colectomy of the splenic flexure	N/A	N/A	Improved
13	[58]	53	M	N/A	None	Celiac, SMA, splenic	Aneurysm in splenic, celiac, SMA, dissection in the celiac.	Aneurysm in the celiac, splenic, and SMA	N/A	Embolization with coil and aortic stent graft	N/A	N/A	Improved

No.	Ref.	Age	Sex		Symptoms	Arteries	Clinical findings	Angiographic findings	Histology	Treatment		Medication	Outcome
14	[59]	51	M	N/A	Abdominal pain, shock	SMA, IMA	Abdominal hemorrhage	Active bleeding from SMA	N/A	Embolization and ligation of the branches of the SMA	N/A	Warfarin	Improved
15	[60]	29	F	N/A	Hypertension	SMA, RA, hepatic	Renal cortical nephrograms	Scattered microaneurysms in SMA, RA, hepatic artery	Segmental lesions of the media with loss of smooth muscle cells	Anti-coagulants	N/A	Warfarin	Improved
16	[61]	55	F	N/A	Abdominal pain	Celiac, SMA, hepatic, splenic	Unremarkable in vessels	Aneurysms in celiac, SMA, hepatic, splenic artery	N/A	Anti-coagulants	N/A	Warfarin followed by aspirin	Improved
17	[62]	56	M	N/A	Abdominal pain, shock	SMA	Aneurysm in MCA, SMA dissection	Saccular aneurysms in the MCA; dissections in the SMA	N/A	Embolization with coil	N/A	N/A	Improved
18	[63]	64	F	N/A	Abdominal pain, back pain, nausea	SMA, IMA, hepatic	Hematoma in the anterior pararenal space inferior to pancreatic tail; bleeding from aneurysm	Multiple aneurysms in the SMA, IMA, hepatic artery	N/A	Conservative	N/A	N/A	Improved
19	[64]	60	F	Hypoxia	Hypoxia, hypotension, cardiopulmonary arrest	SMA	Large hematoma in the retroperitoneal and intraperitoneal space; SMA aneurysm	Aneurysms and beaded appearance in the SMA	N/A	Conservative	N/A	N/A	Improved
20	[65]	36	M	N/A	Abdominal pain	Celiac, hepatic, anterior inferior pancreaticoduodenal artery	Stenosis and aneurysms in anterior inferior pancreatic duodenal artery	Aneurysms and beaded like appearance in the anterior inferior pancreaticoduodenal artery	N/A	Embolization with coil	N/A	N/A	Improved
21	[66]	47	M	N/A	Loss of consciousness, headache, abdominal pain	SMA	SAH, massive intraperitoneal hematoma	Beaded like appearance in SMA; dissection in VA	Medial islands and medial degenerations in SMA	Embolization with coil for VA and SMA. Surgical resection of part of middle colic artery and descending colon.	N/A	N/A	Improved
22	[67]	79	M	N/A	Abdominal pain, hypotension	IMA	Active bleeding from IMA and hemorrhage	N/A	Reduplication of the internal elastic lamina with arterial dissection within the tunica media and thrombus at the site of rupture	Surgical resection of left colic artery	N/A	N/A	
23	[68]	40	M	N/A	Abdominal pain	Celiac, SMA	Extensive dissection of SMA with the thrombotic occlusion. stenosis and dilation of celiac artery	N/A	N/A	Conservative	N/A	N/A	Improved
24	[69]	32	M	N/A	Abdominal pain	IMA, RA	Aneurysm in renal and IMA, massive amount of hemorrhage	Stenosis and aneurysm in the RA	Media shows myxoid degeneration in the outer one-third adjacent to the adventitia	Surgical hemostasis and left hemicolectomy	Yes, N/A	N/A	Improved
25	[70]	58	M	N/A	Abdominal pain	SMA	Mesenteric hematoma and right inguinal hernia with unremarkable small bowel	Beaded like appearance in SMA	N/A	Immunosuppressive therapy	N/A	N/A	Improved

| 26 | [71] | 57 | M | N/A | M | Hypertension, abdominal pain | SMA | Arterial dissection with luminal stenosis and aneurysm formation at the distal portion of the SMA | Segmental dilatation, aneurysm in the SMA | Vacuolization and decrease in the number of vascular smooth muscles | Aneurysmectomy and bowel resection | N/A | Ca-blocker | N/A | Improved |

M: Male; F: Female; N/A: Data not applicable; SMA: Superior mesenteric artery; IMA: Inferior mesenteric artery; RA: Renal artery; CT: Computed tomography; MCA: Middle cerebral artery.

coagulation therapy is uncommon, and only a few cases have been reported to date[59-61]. In addition, given it is a noninflammatory disorder, no evidence of efficacy in use of anti-inflammatory agents or immunosuppressive agents has been reported. However, SAM has been treated with these agents when the differential diagnosis from the other arteritis was difficult[70].

For patients presenting acutely with intra-abdominal hemorrhage, patients are treated with emergent catheter angiography, endovascular intervention, or surgical treatment[73].

Shenouda reported that coil embolization was the most common endovascular intervention and was reported as successful in 88% of patients, with no mortality, whereas the open surgical approach was associated with a 9% mortality rate[72]. In our patient summary, open surgery was most commonly performed, and this was performed on 13 patients (50%). Endovascular intervention was performed on eight patients (31%), and anti-coagulation therapy was administered to two patients (7.7%), including warfarin and aspirin administration. Anti-hypertensive therapy was administered to one patient with Ca-blocker.

Prognosis

Although the prognosis of the disease is reported to be good when managed appropriately[72], SAM can be fatal when ruptured[49,73]. Therefore, a careful diagnosis and appropriate management are essential for this disease entity. Our case summary also showed that although 24 (92%) patients improved, 2 (7.7%) patients died, 1 having had a large hematoma and a ruptured aneurysm in the mesenteric lesion that was revealed upon the emergent surgery.

DISCUSSION

The inner wall of a normal artery is smooth and in the normal condition, blood flows through it without difficulty. The major cause of decreasing the blood flow is atherosclerosis which is due to the deposits of fatty materials, such as cholesterol, developing the thickened arterial walls and stenosis of the vasculatures. These changes cause ischemic changes in the organs fed by the vasculatures and if it occurred in the abdominal mesenteric lesions, the symptoms of severe abdominal pain, ischemic changes of the intestine could be observed leading to lethality. For other vascular diseases including aneurysm, occlusion, and thromboses in the mesenteric lesions could cause severe symptoms and appropriate diagnosis and treatment are essential for managing patients. With the development and improvement of imaging modalities, including CT and magnetic resonance imaging, the frequency of diagnosis of vascular disease in abdominal lesions is increasing. Among these, FMD and SAM are known as noninflammatory, nonatherosclerotic arterial diseases, difficult to be differentially diagnosed from each other. Although various arteries are involved in these diseases, we have focused on the mesenteric areas, reviewing cases in this study and summarizing the clinical characteristics of both disease entities (Table 3).

The histologic findings and the imaging findings of FMD and SAM are similar; for example, Lie proposed that SAM can represent a precursor of certain types of FMD[74]. Slavin and colleagues also proposed that SAM could represent a precursor of FMD, although a part of SAM might remain as unspecified aneurysms[46]. Although these similarities in radiological and histological diagnoses have been reported, the two entities exhibit a different clinical profile in terms of age of onset, sex, distribution of affected arteries, and clinical symptoms. Although FMD affects middle-aged women, there is no predilection for age or sex in SAM[2,73].

Considering the mesenteric lesions, as there are no specific symptoms, a greater knowledge and comprehensive understanding of these diseases are important for appropriate diagnosis and treatment. For example, FMD rarely shows significant symptoms and is frequently associated with symptoms of occlusive disease such as renovascular hypertension, headache, and pulsatile tinnitus. Although FMD does not rupture as often, SAM shows hemorrhages resulting from arterial rupture or dissection

Table 3 Clinical characteristic of the fibromuscular dysplasia and segmental arterial mediolysis

	Fibromuscular dysplasia	Segmental arterial mediolysis
Gender	Female (9:1)[2]	No presentation[74]
Age of presentation	Young to middle age[2]	No preference[74]
Laboratory findings	No serological markers[74]	No serological markers[74]
Risk factors	Smoking and extracranial arteries[4]	Hypoxia and shock or other vasoconstrictor stimuli[47]
Vascular distribution	Renal and extracranial arteries[4]	Celiac and mesenteric arteries[48]
CT	Alternating stenosis and aneurysms, less commonly dissections[38]	Dissections with alternating stenosis and aneurysms, dissecting aneurysms[48]
Angiography	Beaded aneurysmal appearance (string-of-beads)[38]	Beaded aneurysmal appearance (string-of-beads)[38]
Pathology	Fibrous or fibromuscular thickening of the arterial wall[38]	Vecuolization and lysis of the outer media[47]
Symptoms	Renovascular hypertension, Headache, Pulsatile tinnitus[4]	Acute abdominal pain, Intraperitoneal bleeding[47]
Treatment	Anti-platelet therapy and anti-hypertensive therapy. Balloon angioplasty and stenting[45]	Anti-hypertensive therapy and endovascular management, surgical management[74]

CT: Computed tomography.

from the weakened arterial wall[4,46] and is therefore symptomatic with acute abdominal and flank pain.

CONCLUSION

Mesenteric vascular diseases are rare compared with other disease entities in lesions; therefore, clinical information is insufficient and clinical trials to develop the standard therapy are lacking. Therefore, an accumulation of cases and a summary of the clinical characteristics of reported cases are important. For this purpose, we have summarized the characteristics of FMD and SAM in abdominal lesions. This information could help physicians to appropriately diagnose and treat cases, including consultation with interventional radiologists and surgeons.

REFERENCES

1   **Harrison EG Jr**, McCormack LJ. Pathologic classification of renal arterial disease in renovascular hypertension. *Mayo Clin Proc* 1971; **46**: 161-167 [PMID: 5553126]

2   **Olin JW**, Froehlich J, Gu X, Bacharach JM, Eagle K, Gray BH, Jaff MR, Kim ES, Mace P, Matsumoto AH, McBane RD, Kline-Rogers E, White CJ, Gornik HL. The United States Registry for Fibromuscular Dysplasia: results in the first 447 patients. *Circulation* 2012; **125**: 3182-3190 [PMID: 22615343 DOI: 10.1161/CIRCULATIONAHA.112.091223]

3   **Sang CN**, Whelton PK, Hamper UM, Connolly M, Kadir S, White RI, Sanders R, Liang KY, Bias W. Etiologic factors in renovascular fibromuscular dysplasia. A case-control study. *Hypertension* 1989; **14**: 472-479 [PMID: 2680961 DOI: 10.1161/01.HYP.14.5.472]

4   **Olin JW**, Gornik HL, Bacharach JM, Biller J, Fine LJ, Gray BH, Gray WA, Gupta R, Hamburg NM, Katzen BT, Lookstein RA, Lumsden AB, Newburger JW, Rundek T, Sperati CJ, Stanley JC; American Heart Association Council on Peripheral Vascular Disease; American Heart Association Council on Clinical Cardiology; American Heart Association Council on Cardiopulmonary, Critical Care, Perioperative and Resuscitation; American Heart Association Council on Cardiovascular Disease in the Young; American Heart Association Council on Cardiovascular Radiology and Intervention; American Heart Association Council on Epidemiology and Prevention; American Heart Association Council on Functional Genomics and Translational Biology; American Heart Association Council for High Blood Pressure Research; American Heart Association Council on the Kidney in Cardiovascular Disease;

American Heart Association Stroke Council. Fibromuscular dysplasia: state of the science and critical unanswered questions: a scientific statement from the American Heart Association. *Circulation* 2014; **129**: 1048-1078 [PMID: 24548843 DOI: 10.1161/01.cir.0000442577.96802.8c]

5   **Aboumrad MH**, Fine G, Horn RC Jr. Intimal hyperplasia of small mesenteric arteries. Occlusive, with infarction of the intestine. *Arch Pathol* 1963; **75**: 196-200 [PMID: 14010713]

6   **Ripley HR**, Levin SM. Abdominal angina associated with fibromuscular hyperplasia of the celiac and superior mesenteric arteries. *Angiology* 1966; **17**: 297-310 [PMID: 5936946 DOI: 10.1177/000331976601700506]

7   **Wylie EJ**, Binkley FM, Palubinskas AJ. Extrarenal fibromuscular hyperplasia. *Am J Surg* 1966; **112**: 149-155 [PMID: 5911218 DOI: 10.1016/0002-9610(66)90002-X]

8   **Claiborne TS**. Fibromuscular hyperplasia. Report of a case with involvement of multiple arteries. *Am J Med* 1970; **49**: 103-105 [PMID: 5431471 DOI: 10.1016/S0002-9343(70)80118-8]

9   **Lie JT**, Kim HS. Fibromuscular dysplasia of the superior mesenteric artery and coexisting cerebral berry aneurysms. *Angiology* 1977; **28**: 256-260 [PMID: 869283 DOI: 10.1177/000331977702800405]

10 **Rybka SJ**, Novick AC. Concomitant carotid, mesenteric and renal artery stenosis due to primary intimal fibroplasia. *J Urol* 1983; **129**: 798-800 [PMID: 6842706 DOI: 10.1016/S0022-5347(17)52369-1]

11 **Foissy P**, Fabre M, Lebaleur A, Buffet C, Frileux C, Etienne JP. [Aneurysm of the trunk of the superior mesenteric artery and polyaneurysmal disease of the right paracolic arcade of fibromuscular hyperplasia type. A case]. *Ann Med Interne* (Paris) 1984; **135**: 530-532 [PMID: 6517425]

12 **den Butter G**, van Bockel JH, Aarts JC. Arterial fibrodysplasia: rapid progression complicated by rupture of a visceral aneurysm into the gastrointestinal tract. *J Vasc Surg* 1988; **7**: 449-453 [PMID: 3258042 DOI: 10.1016/0741-5214(88)90445-4]

13 **Salmon PJ**, Allan JS. An unusual case of fibromuscular dysplasia. *J Cardiovasc Surg* (Torino) 1988; **29**: 756-757 [PMID: 3209621]

14 **Insall RL**, Chamberlain J, Loose HW. Fibromuscular dysplasia of visceral arteries. *Eur J Vasc Surg* 1992; **6**: 668-672 [PMID: 1451828 DOI: 10.1016/S0950-821X(05)80849-7]

15 Case records of the Massachusetts General Hospital. Weekly clinicopathological exercises. Case 9-1995. A 60-year-old man with hypertrophic cardiomyopathy and ischemic colitis. *N Engl J Med* 1995; **332**: 804-810 [PMID: 7862185 DOI: 10.1056/NEJM199503233321208]

16 **Stokes JB**, Bonsib SM, McBride JW. Diffuse intimal fibromuscular dysplasia with multiorgan failure. *Arch Intern Med* 1996; **156**: 2611-2614 [PMID: 8951305 DOI: 10.1001/archinte.1996.00440210139014]

17 **Yamaguchi R**, Yamaguchi A, Isogai M, Hori A, Kin Y. Fibromuscular dysplasia of the visceral arteries. *Am J Gastroenterol* 1996; **91**: 1635-1638 [PMID: 8759676]

18   **Lee EK**, Hecht ST, Lie JT. Multiple intracranial and systemic aneurysms associated with infantile-onset arterial fibromuscular dysplasia. *Neurology* 1998; **50**: 828-829 [PMID: 9521295 DOI: 10.1212/WNL.50.3.828]

19   **Safioleas M**, Kakisis J, Manti C. Coexistence of hypertrophic cardiomyopathy and fibromuscular dysplasia of the superior mesenteric artery. *N Engl J Med* 2001; **344**: 1333-1334 [PMID: 11336027 DOI: 10.1056/NEJM200104263441716]

20   **Horie T**, Seino Y, Miyauchi Y, Saitoh T, Takano T, Ohashi A, Yamada N, Tamura K, Yamanaka N. Unusual petal-like fibromuscular dysplasia as a cause of acute abdomen and circulatory shock. *Jpn Heart J* 2002; **43**: 301-305 [PMID: 12227706 DOI: 10.1536/jhj.43.301]

21   **Kojima A**, Shindo S, Kubota K, Iyori K, Ishimoto T, Kobayashi M, Tada Y. Successful surgical treatment of a patient with multiple visceral artery aneurysms due to fibromuscular dysplasia. *Cardiovasc Surg* 2002; **10**: 157-160 [PMID: 11888746 DOI: 10.1016/S0967-2109(01)00111-9]

22   **Felton TW**, Drewe E, Jivan S, Hall RI, Powell RJ. A rare case of shock. *Ann Rheum Dis* 2003; **62**: 705-706 [PMID: 12860723 DOI: 10.1136/ard.62.8.705]

23   **Guill CK**, Benavides DC, Rees C, Fenves AZ, Burton EC. Fatal mesenteric fibromuscular dysplasia: a case report and review of the literature. *Arch Intern Med* 2004; **164**: 1148-1153 [PMID: 15159274 DOI: 10.1001/archinte.164.10.1148]

24   **Mertens J**, Daenens K, Fourneau I, Marakbi A, Nevelsteen A. Fibromuscular dysplasia of the superior mesenteric artery--case report and review of the literature. *Acta Chir Belg* 2005; **105**: 523-527 [PMID: 16315839 DOI: 10.1080/00015458.2005.11679773]

25   **Rodriguez Urrego PA**, Flanagan M, Tsai WS, Rezac C, Barnard N. Massive gastrointestinal bleeding: an unusual case of asymptomatic extrarenal, visceral, fibromuscular dysplasia. *World J Gastroenterol* 2007; **13**: 5771-5774 [PMID: 17963307 DOI: 10.3748/wjg.v13.i43.5771]

26   **Chaturvedi R**, Vaideeswar P, Joshi A, Pandit S. Unusual mesenteric fibromuscular dysplasia a rare cause for chronic intestinal ischaemia. *J Clin Pathol* 2008; **61**: 237 [PMID: 18223099 DOI: 10.1136/jcp.2007.049569]

27   **Malagò R**, D'Onofrio M, Mucelli RP. Fibromuscular dysplasia: noninvasive evaluation of unusual case of renal and mesenteric involvement. *Urology* 2008; **71**: 755.e13-755.e15 [PMID: 18313108 DOI: 10.1016/j.urology.2007.10.061]

28   **Veraldi GF**, Zecchinelli MP, Furlan F, Genco B, Minicozzi AM, Segattini C, Pacca R. Mesenteric revascularisation in a young patient with antiphospholipid syndrome and fibromuscular dysplasia: report of a case and review of the literature. *Chir Ital* 2009; **61**: 659-665 [PMID: 20380275]

29   **Kimura K**, Ohtake H, Kato H, Yashiki N, Tomita S, Watanabe G. Multivisceral fibromuscular dysplasia: an unusual case of renal and superior mesenteric involvement. *Ann Vasc Dis* 2010; **3**: 152-156 [PMID: 23555404]

30   **Senadhi V**. A rare cause of chronic mesenteric ischemia from fibromuscular dysplasia: a case report. *J Med Case Rep* 2010; **4**: 373 [PMID: 21092091 DOI: 10.1186/1752-1947-4-373]

31   **Sugiura T**, Imoto K, Uchida K, Yanagi H, Machida D, Okiyama M, Yasuda S, Takebayashi S. Fibromuscular dysplasia associated with simultaneous spontaneous dissection of four peripheral arteries in a 30-year-old man. *Ann Vasc Surg* 2011; **25**: 838.e9-838.11 [PMID: 21616635 DOI: 10.1016/j.avsg.2011.02.018]

32   **Dolak W**, Maresch J, Kainberger F, Wrba F, Müller Ch. Fibromuscular dysplasia mimicking Crohn's disease over a period of 23 years. *J Crohns Colitis* 2012; **6**: 354-357 [PMID: 22405173 DOI: 10.1016/j.crohns.2011.09.012]

33   **Patel NC**, Palmer WC, Gill KR, Wallace MB. A case of mesenteric ischemia secondary to Fibromuscular Dysplasia (FMD) with a positive outcome after intervention. *J Interv Gastroenterol* 2012; **2**: 199-201 [PMID: 23687610 DOI: 10.4161/jig.23747]

34   **Sekar N**, Shankar R. Fibromuscular dysplasia with multiple visceral artery involvement. *J Vasc Surg* 2013; **57**: 1401 [PMID: 23601593 DOI: 10.1016/j.jvs.2011.12.079]

35   **Mitchell A**, Caty V, Bendavid Y. Massive mesenteric panniculitis due to fibromuscular dysplasia of the inferior mesenteric artery: a case report. *BMC Gastroenterol* 2015; **15**: 71 [PMID: 26100669 DOI: 10.1186/s12876-015-0303-5]

36   **Yamada M**, Nakada TA, Idoguchi K, Matsuoka T. Fibromuscular dysplasia presenting as hemorrhagic shock due to spontaneous rupture of a right gastroepiploic artery aneurysm. *Am J Emerg Med* 2016; **34**: 677.e3-677.e5 [PMID: 26166380 DOI: 10.1016/j.ajem.2015.06.054]

37   **Erwin PA**, Blas JV, Gandhi S, Romero ME, Gray BH. Images in Vascular Medicine. Visceral fibromuscular dysplasia in a patient with chronic abdominal pain. *Vasc Med* 2016; **21**: 170-171 [PMID: 26675330 DOI: 10.1177/1358863X15619242]

38   **Brinza EK**, Gornik HL. Fibromuscular dysplasia: Advances in understanding and management. *Cleve Clin J Med* 2016; **83**: S45-S51 [PMID: 27861117 DOI: 10.3949/ccjm.83.s2.06]

39   **Kincaid OW**, Davis GD, Hallermann FJ, Hunt JC. Fibromuscular dysplasia of the renal arteries. Arteriographic features, classification, and observations on natural history of the disease. *Am J Roentgenol Radium Ther Nucl Med* 1968; **104**: 271-282 [PMID: 5685786 DOI: 10.2214/ajr.104.2.271]

40   **Lüscher TF**, Lie JT, Stanson AW, Houser OW, Hollier LH, Sheps SG. Arterial fibromuscular dysplasia. *Mayo Clin Proc* 1987; **62**: 931-952 [PMID: 3309488 DOI: 10.1161/01.STR.13.1.46]

41   **Alimi Y**, Mercier C, Péllissier JF, Piquet P, Tournigand P. Fibromuscular disease of the renal artery: a new histopathologic classification. *Ann Vasc Surg* 1992; **6**: 220-224 [PMID: 1610652 DOI: 10.1007/BF02000266]

42   **Mettinger KL**, Ericson K. Fibromuscular dysplasia and the brain. I. Observations on angiographic, clinical and genetic characteristics. *Stroke* 1982; **13**: 46-52 [PMID: 7064180]

43   **Mettinger KL**. Fibromuscular dysplasia and the brain. II. Current concept of the disease. *Stroke* 1982; **13**: 53-58 [PMID: 7039003 DOI: 10.1161/01.STR.13.1.53]

44   **Weinberg I**, Gu X, Giri J, Kim SE, Bacharach MJ, Gray BH, Katzen BT, Matsumoto AH, Chi YW, Rogers KR, Froehlich J, Olin JW, Gornik HL, Jaff MR. Anti-platelet and anti-hypertension medication use in patients with fibromuscular dysplasia: Results from the United States Registry for Fibromuscular Dysplasia. *Vasc Med* 2015; **20**: 447-453 [PMID: 25964292 DOI: 10.1177/1358863X15584982]

45   **Slavin RE**, Gonzalez-Vitale JC. Segmental mediolytic arteritis: a clinical pathologic study. *Lab Invest* 1976; **35**: 23-29 [PMID: 940319]

46   **Slavin RE**, Saeki K, Bhagavan B, Maas AE. Segmental arterial mediolysis: a precursor to fibromuscular dysplasia? *Mod Pathol* 1995; **8**: 287-294 [PMID: 7617656]

47   **Baker-LePain JC**, Stone DH, Mattis AN, Nakamura MC, Fye KH. Clinical diagnosis of segmental arterial mediolysis: differentiation from vasculitis and other mimics. *Arthritis Care Res* (Hoboken) 2010; **62**: 1655-1660 [PMID: 20662047 DOI: 10.1002/acr.20294]

48   **Sakano T**, Morita K, Imaki M, Ueno H. Segmental arterial mediolysis studied by repeated angiography. *Br J Radiol* 1997; **70**: 656-658 [PMID: 9227264 DOI: 10.1259/bjr.70.834.9227264]

49   **Rengstorff DS**, Baker EL, Wack J, Yee LF. Intra-abdominal hemorrhage caused by segmental arterial mediolysis of the inferior mesenteric artery: report of a case. *Dis Colon Rectum* 2004; **47**: 769-772 [PMID: 15054678 DOI: 10.1007/s10350-003-0103-9]

50   **Chino O**, Kijima H, Shibuya M, Yamamoto S, Kashiwagi H, Kondo Y, Makuuchi H. A case report: spontaneous rupture of dissecting aneurysm of the middle colic artery. *Tokai J Exp Clin Med* 2004; **29**: 155-158 [PMID: 15717485]

51   **Basso MC**, Flores PC, de Azevedo Marques A, de Souza GL, D'Elboux Guimarães Brescia M, Campos CR, de Cleva R, Saldiva PH, Mauad T. Bilateral extensive cerebral infarction and mesenteric ischemia associated with segmental arterial mediolysis in two young women. *Pathol Int* 2005; **55**: 632-638 [PMID: 16185293 DOI: 10.1111/j.1440-1827.2005.01881.x]

52   **Obara H**, Matsumoto K, Narimatsu Y, Sugiura H, Kitajima M, Kakefuda T. Reconstructive surgery for segmental arterial mediolysis

involving both the internal carotid artery and visceral arteries. *J Vasc Surg* 2006; **43**: 623-626 [PMID: 16520184 DOI: 10.1016/j.jvs.2005.11.033]

53   **Abdelrazeq AS**, Saleem TB, Nejim A, Leveson SH. Massive hemoperitoneum caused by rupture of an aneurysm of the marginal artery of Drummond. *Cardiovasc Intervent Radiol* 2008; **31** Suppl 2: S108-S110 [PMID: 17710481 DOI: 10.1007/s00270-007-9117-3]

54   **Shimohira M**, Ogino H, Sasaki S, Ishikawa K, Koyama M, Watanabe K, Shibamoto Y. Transcatheter arterial embolization for segmental arterial mediolysis. *J Endovasc Ther* 2008; **15**: 493-497 [PMID: 18729557 DOI: 10.1583/08-2384.1]

55   **Hashimoto T**, Deguchi J, Endo H, Miyata T. Successful treatment tailored to each splanchnic arterial lesion due to segmental arterial mediolysis (SAM): report of a case. *J Vasc Surg* 2008; **48**: 1338-1341 [PMID: 18971044 DOI: 10.1016/j.jvs.2008.05.056]

56   **Hirokawa T**, Sawai H, Yamada K, Wakasugi T, Takeyama H, Ogino H, Tsurusaki M, Arai Y. Middle-colic artery aneurysm associated with segmental arterial mediolysis, successfully managed by transcatheter arterial embolization: report of a case. *Surg Today* 2009; **39**: 144-147 [PMID: 19198994 DOI: 10.1007/s00595-008-3811-x]

57   **Fujiwara Y**, Takemura M, Yoshida K, Morimura K, Inoue T. Surgical resection for ruptured aneurysm of middle colic artery caused by segmental arterial mediolysis: a case report. *Osaka City Med J* 2010; **56**: 47-52 [PMID: 21466129]

58   **Obara H**, Matsubara K, Inoue M, Nakatsuka S, Kuribayashi S, Kitagawa Y. Successful endovascular treatment of hemosuccus pancreaticus due to splenic artery aneurysm associated with segmental arterial mediolysis. *J Vasc Surg* 2011; **54**: 1488-1491 [PMID: 21715127 DOI: 10.1016/j.jvs.2011.04.053]

59   **Tameo MN**, Dougherty MJ, Calligaro KD. Spontaneous dissection with rupture of the superior mesenteric artery from segmental arterial mediolysis. *J Vasc Surg* 2011; **53**: 1107-1112 [PMID: 21276678 DOI: 10.1016/j.jvs.2010.11.034]

60   **Filippone EJ**, Foy A, Galanis T, Pokuah M, Newman E, Lallas CD, Gonsalves CF, Farber JL. Segmental arterial mediolysis: report of 2 cases and review of the literature. *Am J Kidney Dis* 2011; **58**: 981-987 [PMID: 21872379 DOI: 10.1053/j.ajkd.2011.05.031]

61   **Taira S**, Katori H, Matsuda Y, Tani I. [Case report: a case of segmental arterial mediolysis (SAM) with bilateral renal infarction, superior mesenteric aneurysm and splenic aneurysm]. *Nihon Naika Gakkai Zasshi* 2011; **100**: 1966-1968 [PMID: 21863772 DOI: 10.2169/naika.100.1966]

62   **Yoo BR**, Han HY, Cho YK, Park SJ. Spontaneous rupture of a middle colic artery aneurysm arising from superior mesenteric artery dissection: Diagnosis by color Doppler ultrasonography and CT angiography. *J Clin Ultrasound* 2012; **40**: 255-259 [PMID: 22457222 DOI: 10.1002/jcu.21906]

63   **Horsley-Silva JL**, Ngamruengphong S, Frey GT, Paz-Fumagalli R, Lewis MD. Segmental arterial mediolysis: a case of mistaken hemorrhagic pancreatitis and review of the literature. *JOP* 2014; **15**: 72-77 [PMID: 24413790 DOI: 10.6092/1590-8577/2036]

64   **Gulati G**, Ware A. Segmental arterial mediolysis: a rare non-inflammatory cause of mesenteric bleeding. *BMJ Case Rep* 2015; **2015**: [PMID: 26135493 DOI: 10.1136/bcr-2015-210344]

65   **Fujinaga J**, Kuriyama A. Segmental Arterial Mediolysis. *J Emerg Med* 2016; **51**: 732-733 [PMID: 27670290 DOI: 10.1016/j.jemermed.2016.07.090]

66   **Shinoda N**, Hirai O, Mikami K, Bando T, Shimo D, Kuroyama T, Matsumoto M, Itoh T, Kuramoto Y, Ueno Y. Segmental Arterial Mediolysis Involving Both Vertebral and Middle Colic Arteries Leading to Subarachnoid and Intraperitoneal Hemorrhage. *World Neurosurg* 2016; **88**: 694.e5-694.10 [PMID: 26724638 DOI: 10.1016/j.wneu.2015.12.058]

67   **Galketiya K**, Llewellyn H, Liang X. Spontaneous haemoperitoneum due to segmental arterial mediolysis and rupture of the left colic artery. *ANZ J Surg* 2016; **86**: 201-202 [PMID: 24801961 DOI: 10.1111/ans.12654]

68   **Kuriyama A**. Segmental arterial mediolysis. *Am J Emerg Med* 2017; **35**: 518.e1-518.e2 [PMID: 27742517 DOI: 10.1016/j.ajem.2016.09.036]

69   **Yoshioka T**, Araki M, Ariyoshi Y, Wada K, Tanaka N, Nasu Y. Successful microscopic renal autotransplantation for left renal aneurysm associated with segmental arterial mediolysis. *J Vasc Surg* 2017; **66**: 261-264 [PMID: 27988157 DOI: 10.1016/j.jvs.2016.09.039]

70   **Japikse RD**, Sevenson JE, Pickhardt PJ, Repplinger MD. Segmental Arterial Mediolysis: An Unusual Case Mistaken to be a Strangulated Hernia. *WMJ* 2017; **116**: 173-176 [PMID: 29323836]

71   **Akuzawa N**, Kurabayashi M, Suzuki T, Yoshinari D, Kobayashi M, Tanahashi Y, Makita F, Saito R. Spontaneous isolated dissection of the superior mesenteric artery and aneurysm formation resulting from segmental arterial mediolysis: a case report. *Diagn Pathol* 2017; **12**: 74 [PMID: 29037200 DOI: 10.1186/s13000-017-0664-x]

72   **Shenouda M**, Riga C, Naji Y, Renton S. Segmental arterial mediolysis: a systematic review of 85 cases. *Ann Vasc Surg* 2014; **28**: 269-277 [PMID: 23988553 DOI: 10.1016/j.avsg.2013.03.003]

73   **Pillai AK**, Iqbal SI, Liu RW, Rachamreddy N, Kalva SP. Segmental arterial mediolysis. *Cardiovasc Intervent Radiol* 2014; **37**: 604-612 [PMID: 24554198 DOI: 10.1007/s00270-014-0859-4]

74   **Lie JT**. Segmental mediolytic arteritis: Not an arteritis but a variant of arterial fibromuscular dysplasia. *Arch Pathol Lab Med* 1992; **116**: 238-241[PMID: 1536608]

Abnormal expression of *HMGB-3* is significantly associated with malignant transformation of hepatocytes

Wen-Jie Zheng, Min Yao, Miao Fang, Li Wang, Zhi-Zhen Dong, Deng-Fu Yao

Wen-Jie Zheng, Miao Fang, Deng-Fu Yao, Research Center of Clinical Medicine, Affiliated Hospital of Nantong University, Nantong 226001, Jiangsu Province, China

Min Yao, Department of Immunology, Medical School of Nantong University, Nantong 226001, Jiangsu Province, China

Li Wang, Department of Medical Informatics, Medical School of Nantong University, Nantong 226001, Jiangsu Province, China

Zhi-Zhen Dong, Department of Diagnostics, Affiliated Hospital of Nantong University, Nantong 226001, Jiangsu Province, China

ORCID number: Wen-Jie Zheng (0000-0002-3073-4596); Min Yao (0000-0002-5473-0186); Miao Fang (0000-0002-3211-6230); Li Wang (0000-0003-2838-9807); Zhi-Zhen Dong (0000-0001-8862-830x); Deng-Fu Yao (0000-0002-3448-7756).

Abstract

AIM

To explore the relationship between dynamic expression of high mobility group box-3 (HMGB3) and malignant transformation of hepatocytes.

METHODS

Expression of HMGB family proteins were observed in rat hepatocarcinogenesis models induced with 2-acety-laminofluorene. Alterations of HMGB3 were analyzed at the mRNA level by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and at the protein level by immunohistochemistry or Western blotting. HMGB3 in human liver cancer tissues were evaluated using bioinformatics databases from GEO, TCGA, and Oncomine. A specific HMGB3-shRNA was used to knock

down HMGB3 expression in order to investigate its effects on proliferation and cell cycle *in vitro* and *in vivo*.

RESULTS
Elevated HMGB3 levels were first reported in hepato-carcinogenesis, with increasing expression from normal liver to cancer. Bioinformatic databases showed that HMGB3 expression in hepatocellular carcinoma tissues was significantly higher than that in normal liver tissues. Higher HMGB3 expression was discovered in liver cancer cells compared with LO2 cells *in vitro*. According to gene set enrichment analysis, HMGB3 mRNA levels were correlated with cell cycle and DNA replication pathways. Knocking down HMGB3 by specific shRNA significantly inhibited proliferation of HepG2 cells by cell cycle arrest and downregulating DNA replication related genes (cyclin B1, FEN1, and PCNA) at the mRNA and protein level. Furthermore, silencing HMGB3 significantly inhibited xenograft tumor growth (measured by Ki67) *in vivo*.

CONCLUSION
HMGB3 is involved in malignant transformation of hepatocytes and could be a useful biomarker for diagnosis and a potential target for therapy of liver cancer.

Key words: Liver cancer; *HMGB-3*; Hepatocarcinogenesis; Proliferation; Tumor growth

Core tip: High mobility group box (HMGB) family proteins were correlated with hepatocellular carcinoma (HCC) development and progression. This current study examined the effects of HMGB3 on HCC both *in vitro* and *in vivo*. Overexpression of HMGB3 was observed in hepatic malignant transformation and HCC tissues in bioinformatic databases. Knockdown of HMGB3 significantly inhibited proliferation, cell cycle, and tumor growth of HCC cells, providing a novel insight for HCC research.

INTRODUCTION
Hepatocellular carcinoma (HCC) is one of the most common and highly aggressive cancers worldwide, with the third most common cause of cancer related death[1]. Various factors can cause HCC, including cirrhosis, infections with Hepatitis B virus (HBV) or HCV, nonalcoholic fatty liver disease (NFALD), diabetes, aflatoxin B1, tobacco, and excessive alcohol consumption. Among them, chronic infections with HBV and HCV account for more than 60% of total HCC cases[2,3]. In past decades, although obvious improvement has been observed in therapeutic approaches, the prognosis of HCC remains poor because of aggressive invasiveness, frequent metastasis, and multi-drug resistance (MDR). Given that multiple genes and signaling pathways play crucial roles in the occurrence and development of HCC, target therapy is a promising approach for HCC treatment[4]. The Food and Drug Administration (FDA) has approved Sorafenib, a multi-kinase inhibitor, as a first-line treatment for advanced HCC. However, the overall effects are partially unsatisfying due to its low response rate and high frequency of adverse events[5,6]. Thus, it is of great importance to identify novel biomarkers for early diagnosis and potential targets against the progression of HCC.

The high mobility group (HMG)-box (HMGB) family belongs to the HMG protein superfamily[7] (HMGA[8], HMGB[9], and HMGN[10]). HMGB consists of four members (HMGB1, HMGB2, HMGB3, and HMGB4) with similar physiology and pathology features. It encodes proteins containing one or more DNA-binding motifs and participates in multiple cellular processes including cell differentiation, migration, and inflammatory-related activities. The HMGB family plays a complex role in carcinogenesis due to its diverse tumorigenic bioactivities in tumors. HBV functionally binds to HMG protein and activates it[11]. Mitochondrial biogenesis mediated by hypoxia promotes HCC growth through interaction between HMGB1 and Toll-like receptor 9[12]. HMGB1 secretion could be stimulated by HBX protein and subsequently enhance HCC metastasis[13]. HMGB1 signaling is also regulated by specific long noncoding RNA[14] or microRNA[15] showing pro- or anti- effects on invasion and metastasis of HCC[16]. Moreover, overexpression of HMGB2 is associated with aggressiveness and prognosis of HCC[17]. However, until now, there is rather less known about the HMGB3 or HMGB4 expression in HCC progression.

HMGB3 is a multifunctional protein with various roles in different cellular compartments and localized in the nucleus, chromosomes, and cytoplasm. It contributes to the balance between self-renewal and differentiation of hematopoietic stem cells, and enhances DNA flexibility to activate gene promoters[18]. Recently, abnormal HMGB3 has been characterized as pro-carcinogenic by promoting tumor growth, proliferation, invasion, and metastasis in several tumors including gastric[19], lung[20], esophageal[21], breast[22], colorectal[23], and urinary bladder[24]. However, the current knowledge concerning the positive and negative effects of HMGB3 on HCC development is not explicit. The aims of this study were to investigate the dynamic HMGB3 expression in hepatocarcinogenesis, bioinformatics databases, HCC cell lines, and a xenograft model, as well as to validate HMGB3 as a diagnostic marker or novel target gene for HCC.

MATERIALS AND METHODS
Rat hepatocarcinogenesis model
Forty Sprague-Dawley rats (4-6 wk old) were provided

by the Experimental Animal Center of Nantong University. Living conditions included a clean environment, 12 h light/dark cycle, and 55% humidity as previously described[25]. The control group (*n* = 10) were fed a normal diet, and the hepatocarcinogenesis group (*n* = 30) were fed a diet with 0.05% 2-acetylaminofluorene (2-AAF, Sigma, United States). The rats were sacrificed at different times depending on their condition. Rat livers were used for pathology, RNA extraction, and quantitative analysis of HMGB expression. Following the determination of morphological changes in the rat livers and hematoxylin and eosin (H&E) staining, the hepatocarcinogenesis group was divided into three subgroups: degeneration (*n* = 6), precancerous (*n* = 6), and HCC (*n* = 6). All procedures *in vivo* were performed according to the guidelines of Animal Care and Use Committee of Nantong University, China.

Cell culture and transfection
Human HCC cells HepG2, SMMC7721, HCCLM3, Huh7, BEL7404 and normal hepatocyte L02 were purchased from Cell Bank of Chinese Academy of Science (Shanghai, China). All cell lines were maintained in Dulbecco modified Eagle medium or RPMI1640 medium supplemented with 10% fetal bovine serum and antibiotics at 37 ℃ in a humidified incubator with 5% CO_2. Three candidate shRNAs were designed by Genema (Shanghai, China). Cell transfection was conducted according to manufacturer's instructions. Briefly, once cells reached 80% confluence, plasmids were gently transfected into cells using a transfection regent kit. After incubation for 12 h, cells were treated with fresh complete medium. The transfection efficiency was observed using a fluorescence microscope after 24 h. shRNA sequences were as follows: shRNA-1, 5'-GGAAAGTTTGATGGTGCAAAG-3'; shRNA-2, 5'-CGATCATATTGTAGTCTCTCA-3'; shRNA-3, 5'-CCTCCCTATAAATGTGGTAGC-3'; and NC-shRNA, 5'-GGAAGACGATGTCCGGGAAAG-3'.

Histopathological examination
Sections of the formalin-fixed paraffin-embedded (FFPE) tissues were deparaffinized in xylene and rehydrated with a series of graded ethanol. After incubation in hematoxylin solution for 15 min, sections were counterstained in eosin solution using the Hematoxylin and Eosin (H&E) Staining Kit (Solarbio, China) according to the manufacturer's instructions. Samples were viewed under a light microscope.

Bioinformatics analysis
In order to analyze the mRNA expression of HMGB3 in more HCC samples, gene expression profiling data from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (GSE-14520, GSE-5364, GSE-77314, and GSE-50579, United States), The Cancer Genome Atlas (TCGA) database, and Oncomine database (United States) were incorporated in this study. All data extracted from bioinformatics databases were presented as $\log_2$ value.

Gene set enrichment analysis
Gene set enrichment analysis (GSEA v2.2) was performed to discover the differences in biological processes and signaling pathways in transcript levels between high and low HMGB3 expression in GSE-14520 and TCGA. Gene sets were obtained from the Molecular Signatures Database (MSigDB). Enrichment scores (ES) were calculated to estimate genes from a pre-defined gene set. The positive enrichment score indicated that the gene set was considered upregulated while negative score meant downregulated. The number of permutations was set to 1000, and $P < 0.05$ was considered significantly enriched.

MTT assay
Cell proliferation was detected with the MTT assays. HepG2 cells transfected with shRNA-1 and NC-shRNA were seeded in the 96-well plate at a concentration of 3000 cells/well. Then, MTT solution (0.5 mg/ml) was added to the appropriate wells for the 1st ,2nd, 3rd, and 4th d. Following a four hour incubation, DMSO was added. Then, the absorbance was detected at a wavelength of 490 nm.

Cell cycle
Cells were collected by trypsin and fixed in 70% methanol for 30 min. Then cells were resuspended in PBS containing 50 μg/mL propidium iodide (Invitrogen, United States) for one hour at room temperature. After that, samples were analyzed by a flow cytometer (BD Biosciences, United States). Percentage of each cycle phase was calculated by Modfit software.

Immunohistochemistry
Immunohistochemical analysis was performed as previously described[25]. In brief, tissue samples were fixed with formalin, embedded in paraffin, and cut into 4 μm thick sections. Following incubation at 70 ℃ for one hour, slides were deparaffinized in xylene and rehydrated with gradient ethanol. Antigen retrieval was conducted using EDTA solution at pH 8.0. After being blocked for one hour, slides were incubated with primary antibody overnight at 4 ℃ and then with the secondary antibody for two hours at room temperature. After that, slides were visualized by DAB, and counterstained by hematoxylin.

Western blotting
Total protein was extracted from cell lysates using RIPA solution according to the manufacturers' instructions and separated by 10% SDS-PAGE. Then the samples were transferred onto a PVDF membrane. After blocking with PBS with 5% BSA, the membranes were incubated with primary antibodies at 1:1000 dilution (HMGB3, R&D, United States; Cyclin B1, FEN1, and PCNA, Abcam, United States; GAPDH, CST, United States) at 4 ℃ overnight. After secondary antibody incubation, the

Table 1 Primers for real-time polymerase chain reaction			
Gene symbol	Species	Primer sequence (5'-3')	Location
HMGB1	Rat	F: GCTGACAAGGCTCGTTATGAA	186-205
		R: CCTTTGATTTTGGGGCGGTA	381-361
HMGB2	Rat	F: CGGGGCAAAATGTCCTCGTA	28-47
		R: ATGGTCTTCCATCTCTCGGAG	155-135
HMGB3	Rat	F: AGGTGACCCCAAGAAACCAAA	9-29
		R: TCAGCAAAATTGACGGGAACC	119-99
HMGB4	Rat	F: AGACCAGCTAAGGCCCAAG	12-30
		R: CCTTTTCGTGCTTTGAGATGGAT	172-150
HMGB3	Human	F: CCAAAGGGCAAGATGTCCG	25-43
		R: TTGACAGGGACCTCTGGGTTT	110-90
CCNB1	Human	F: AATAAGGCGAAGATCAACATGGC	43-65
		R: TTTGTTACCAATGTCCCCAAGAG	153-131
FEN1	Human	F: ATGACATCAAGAGCTACTTTGGC	62-84
		R: GGCGAACAGCAATCAGGAACT	142-122
PCNA	Human	F: CCTGCTGGGATATTAGCTCCA	77-97
		R: CAGCGGTAGGTGTCGAAGC	185-167

HMGB: High mobility group box; FEN1: Flap structure-specific endonuclease 1; PCNA: Proliferating cell nuclear antigen; F: Forward primer; R: Reverse primer.

samples were detected using the ECL detection system (Bio-Rad, United States).

Reverse transcription-quantitative polymerase chain reaction

Total RNA was extracted from tissues using Trizol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized by using a reverse transcription kit (Invitrogen, CA, United States). Quantitative polymerase chain reaction (qPCR) was conducted by using SYBR Premix Ex Taq kit (Takara, Japan) according to the manufacturer's instructions. The relative mRNA expression (normalized to GAPDH) was assessed using the $2^{-\Delta\Delta Ct}$ ($\Delta\Delta C_t = \Delta C_{t[target\ gene]} - \Delta C_{t[GAPDH]}$) analysis method. The primers in this study were presented in Table 1.

Xenograft assay

Male BALB/c nude mice (4-6 wk old) were subcutaneously injected with 2×10^6 HepG2 cells transfected with shRNA-1 or NC-shRNA. Tumor size was measured every four days and calculated according to the formula (Volume= length $\times$ width^2 $\times$ 1/2). Mice were sacrificed at the 30th d after injection. Tumors were weighed and fixed for further immunohistochemistry of HMGB3 and Ki67 (1:50, Abcam, United States). All procedures were approved by Animal care committee of Nantong University.

Statistics

The data in this study are presented as means ± standard deviation (SD) of at least three experiments. Comparisons between groups were performed using Two-tailed Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

RESULTS

Upregulating expression of HMGB3 in hepatocarcinogenesis

According to the morphological alteration and H&E staining, rats were divided into four groups: normal, degeneration, precancerous, and cancerous. The degeneration group was characterized as the granule-like degeneration in the cytoplasm. The precancerous group was characterized as dense nuclear chromatin and high ratio of nucleus to cytoplasm. The cancerous group showed denser nuclear chromatin, upper ratio of nucleus to cytoplasm, and loss of hepatic structure (Figure 1A). Then expression of the HMGB family (HMGB1, HMGB2, HMGB3, and HMGB4) were detected in liver tissues of each group above by RT-qPCR. No statistically significant changes of HMGB1 (Figure 1B) or HMGB2 (Figure 1C) were found among the four groups. Notably, hepatic HMGB3 expression had a significant increase during the transformation from normal hepatocytes to HCC (Figure 1D). HMGB4 expression was downregulated through the progression to HCC (Figure 1E). The upregulation of hepatic HMGB3 at protein level from normal to cancerous group (Figure 1F) were confirmed by IHC staining.

Validation of HMGB3 mRNA by bioinformatics databases

To further verify the HMGB3 expression in human HCC tissues, data from several bioinformatics databases (GEO, TCGA, and Oncomine) of normal livers ($n = 359$) and HCC tissues ($n = 765$) were analyzed. Compared with normal livers, HMGB3 had higher expression in HCC tissues according to the GSE14520 (fold change = 1.896, $t = 11.270$, $P < 0.001$, Figure 2A), GSE5364 (fold change = 1.720, $t = 4.161$, $P = 0.002$, Figure 2B), GSE77314 (fold change = 2.204, $t = 4.473$, $P < 0.001$, Figure 2C), and TCGA database (fold change = 1.709, $t = 9.125$, $P < 0.001$, Figure 2D). Meanwhile, GSE50597 presented upregulated HMGB3 expression at advance stage of HCC in comparison with that at early stage (fold change = 2.054, $t = 3.046$, $P = 0.012$, Figure 2E). Besides, Oncomine database elucidated that higher HMGB3 expression was detected in HCC tissues rather than liver cancer precursor (fold change = 1.469, $t = 2.948$, $P = 0.005$) or normal livers (fold change = 1.795, $t = 3.380$, $P = 0.003$, Figure 2F). Given the observation
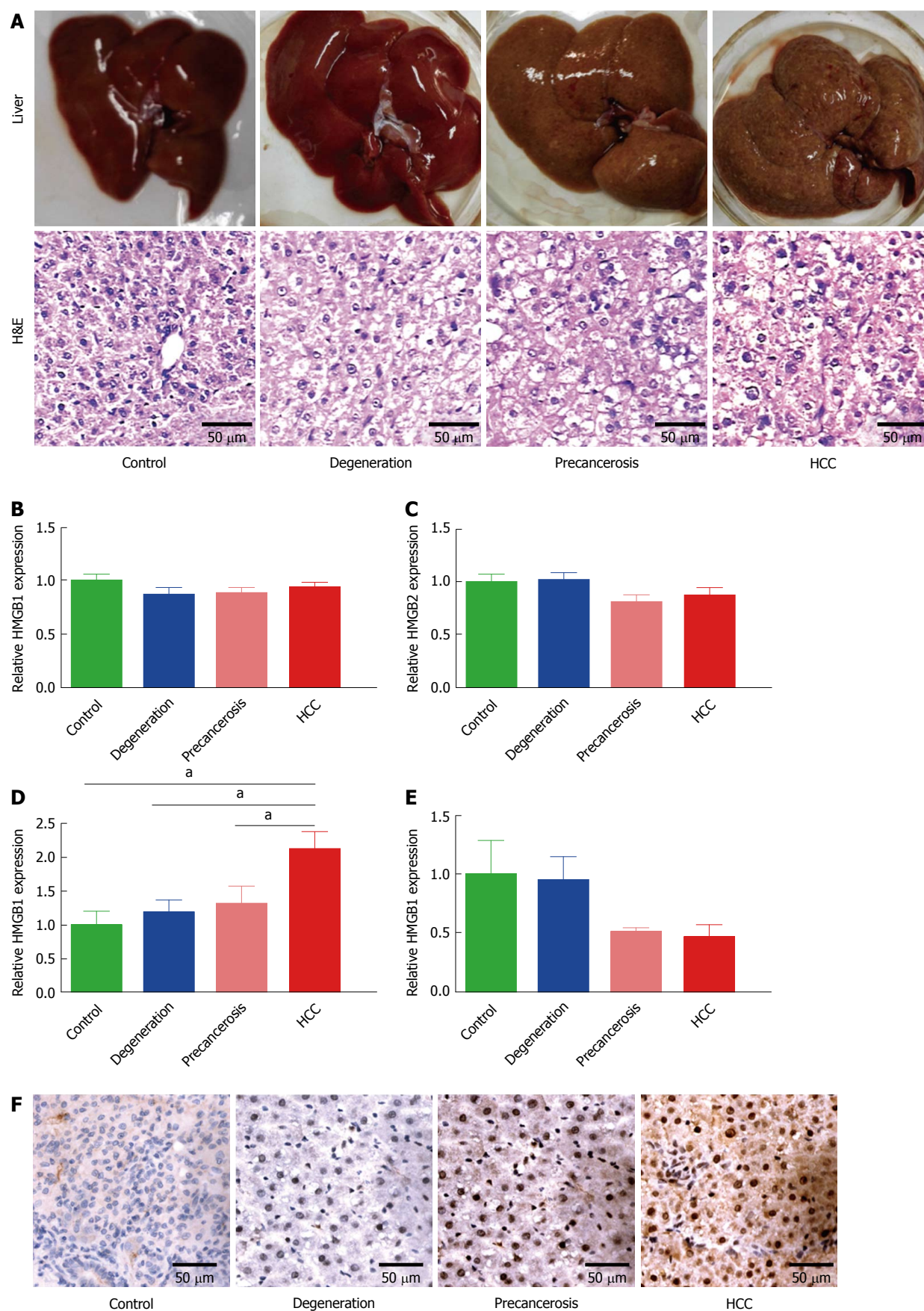
Figure 1 Dynamic upregulation of *HMGB3* in rat hepatocarcinogenesis. Rat hepatocarcinogenesis models were successfully made by consistent 2-AAF intake. A: The dynamic alterations of liver morphology (upper panel) and H&E staining (lower panel) of liver tissues in rat hepatocarcinogenesis. The livers of the rat model, according to the results of rat liver H&E staining, were divided into normal, degeneration, precancerous, and HCC group. B-E: the dynamic alterations of the HMGB family at the mRNA level in models were detected by RT-qPCR. B: HMGB1 mRNA. C: HMGB2 mRNA. D: HMGB3 mRNA. E: HMGB4 mRNA. Each band was presented as a relative value normalized to normal controls (*n* = 6). F: the immunohistochemical staining of rat HMGB3 expression in different groups. [a]*P* < 0.05. 2-AAF: 2-acetylaminofluorene; H&E: Hematoxylin and eosin; HMGB: High mobility group-box; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction.
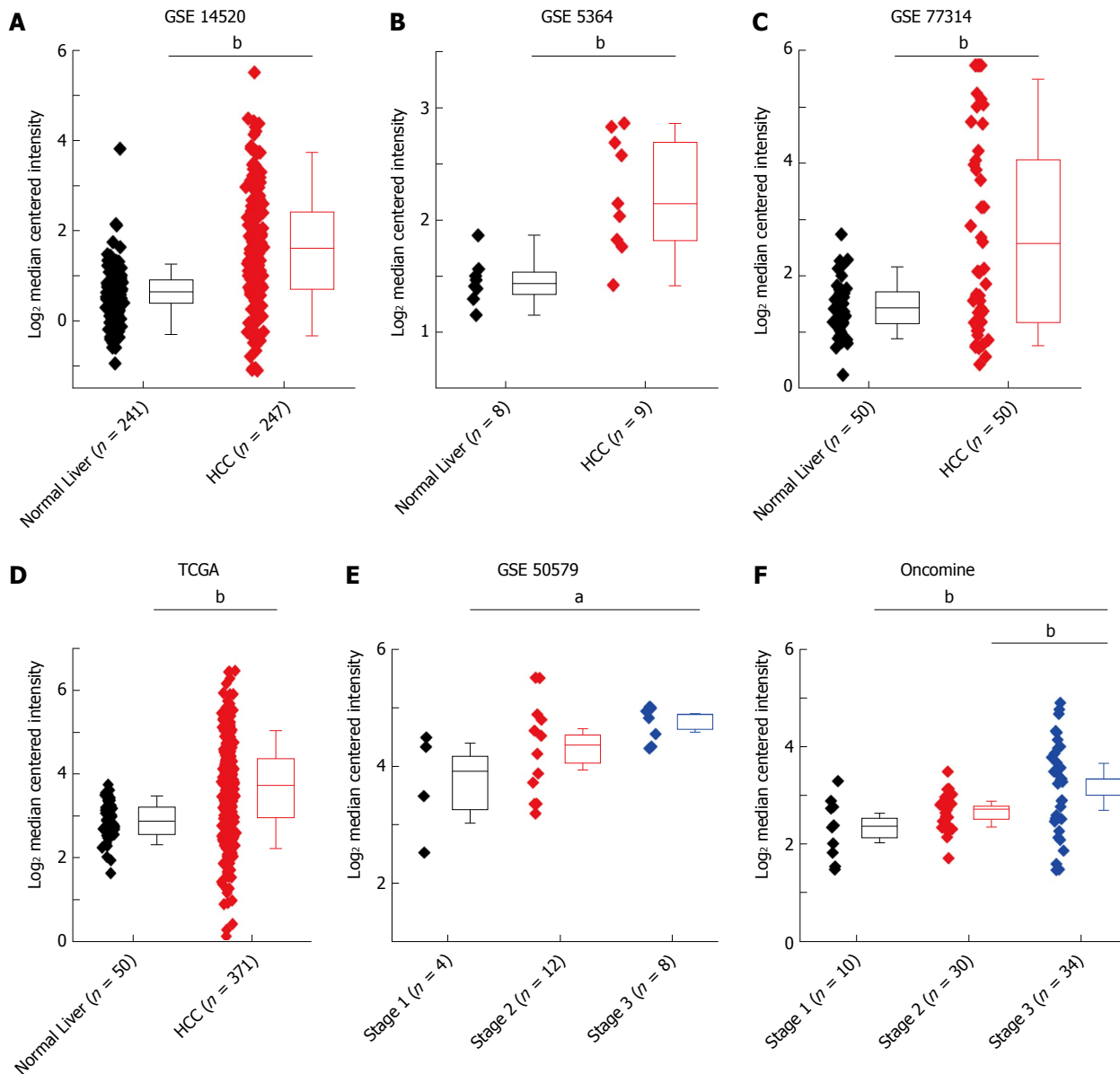
Figure 2 *HMGB3* **mRNA expression related to hepatocellular carcinoma by bioinformatic databases.** Comparative analysis of human normal livers (*n* = 359) and HCC (*n* = 765) tissues from bioinformatics databases suggested that the upregulation of hepatic HMGB3 mRNA might be involved in HCC progression. The data of HMGB3 mRNA in HCC or normal liver were extracted from A: GSE-14520, B: GSE-5364, C: GSE-77314, D: the HMGB3 mRNA in TCGA database, E: GSE-50579, and F: the HMGB3 mRNA in Oncomine database. Values were presented as Log2 median centered intensity. [a]$P < 0.05$; [b]$P < 0.01$.GSE and GEO Series; TCGA: The Cancer Genome Atlas; HCC: Hepatocellular carcinoma.

above, the data suggested that the upregulation of liver HMGB3 mRNA expression might be related to HCC progression.

Knockdown of HMGB3 in HCC cell lines
To further determine the role of HMGB3 in HCC progression, HMGB3 expression was detected and silenced in HCC cell lines. In contrast to normal hepatocyte L02, HMGB3 was overexpressed in HCC cell lines Huh7, HepG2, HCCLM3, SMMC7721, and BEL7404 (Figure 3A and 3B). HepG2 had the highest expression of HMGB3 and was chosen to conduct RNAi using three specific shRNAs with GFP labeling (Figure 3C). Compared with the control and NC-shRNA group, HMGB3 expression was significantly (*P* < 0.001) downregulated with shRNA-1 at

the mRNA (Figure 3D) and protein levels (Figure 3E and F).

Silencing HMGB3 inhibited proliferation and regulated cell cycle of HCC cells
Gene set enrichment analysis was performed to sort the pathways enriched in distinct phenotype labels according to HMGB3 levels. In both of GSE14520 and TCGA, high expression of HMGB3 was correlated with cell cycle and DNA replication (Figure 4A). Thus, the proliferation activity and cell cycle were studied in HMGB3-knockdown HepG2 cells. As shown in Figure 4B, knockdown of HMGB3 using shRNA-1 significantly inhibited the proliferation of HepG2 cells. Furthermore, silencing HMGB3 could lead to an obvious arrest of HepG2 cells in the G1
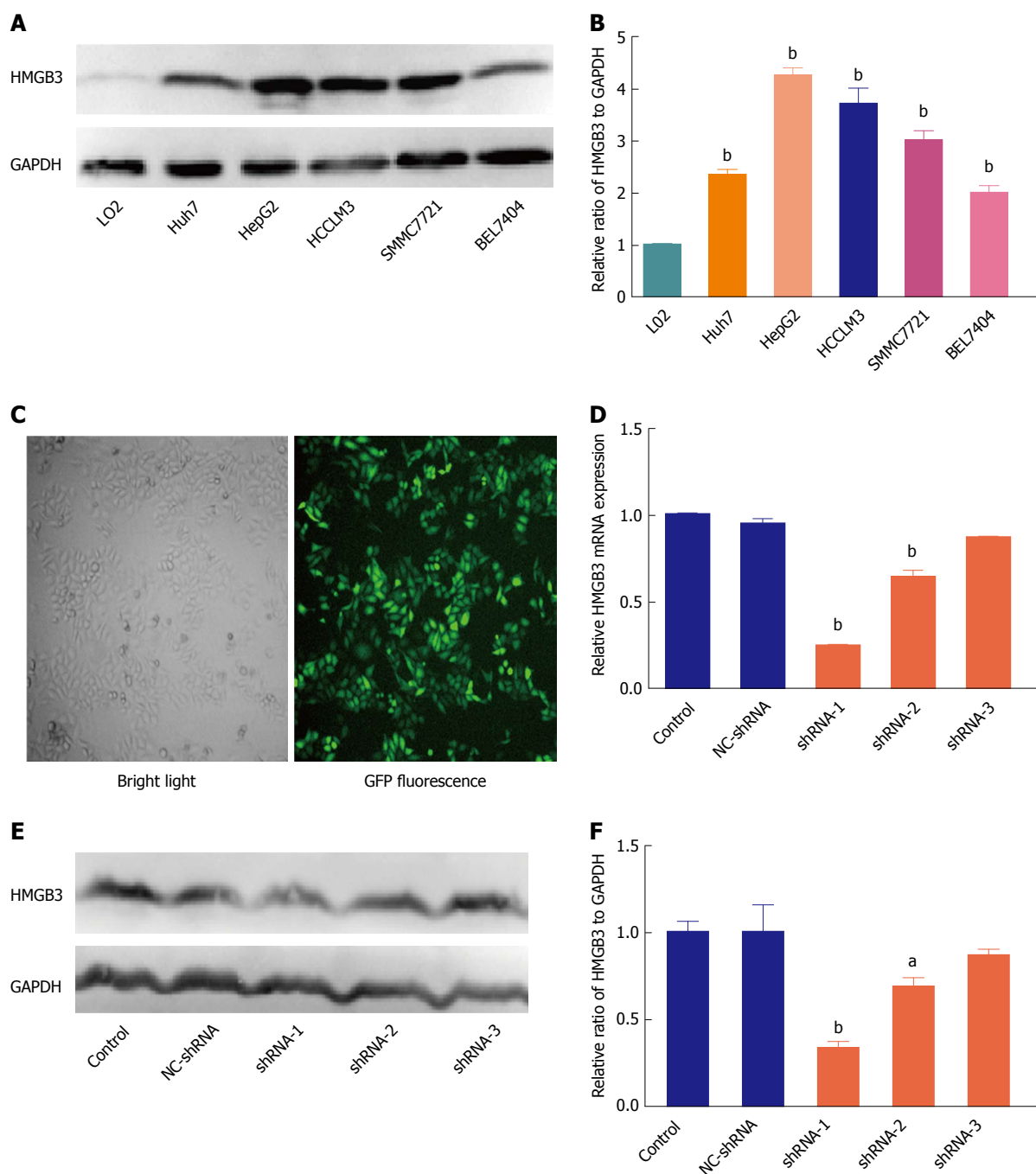
Figure 3 Silencing *HMGB3* in hepatocellular carcinoma cell lines. A: The protein expression of HMGB3 was detected in different HCC cell lines (Huh7, HepG2, HCCLM3, SMMC7721, and BEL7404), and normal hepatocytes L02 using western blotting. GAPDH was used as an internal reference. B: Each bar represents the corresponding intensity in A normalized to GAPDH. C: Representative morphology of HepG2 cells transfected with GFP-labeling shRNA in bright light and fluorescence. D: RT-qPCR was performed to detect HMGB3 mRNA levels in HepG2 cells transfected with different shRNAs and control. Relative value of HMGB3 was calculated according to the $2^{-\Delta\Delta Ct}$ method. E: Western blotting was conducted to analyze the HMGB3 protein expression in cells of the shRNA-transfected group and control group. F: Each bar represents the corresponding intensity in E normalized to GAPDH. $^{a}P < 0.05$; $^{b}P < 0.01$.
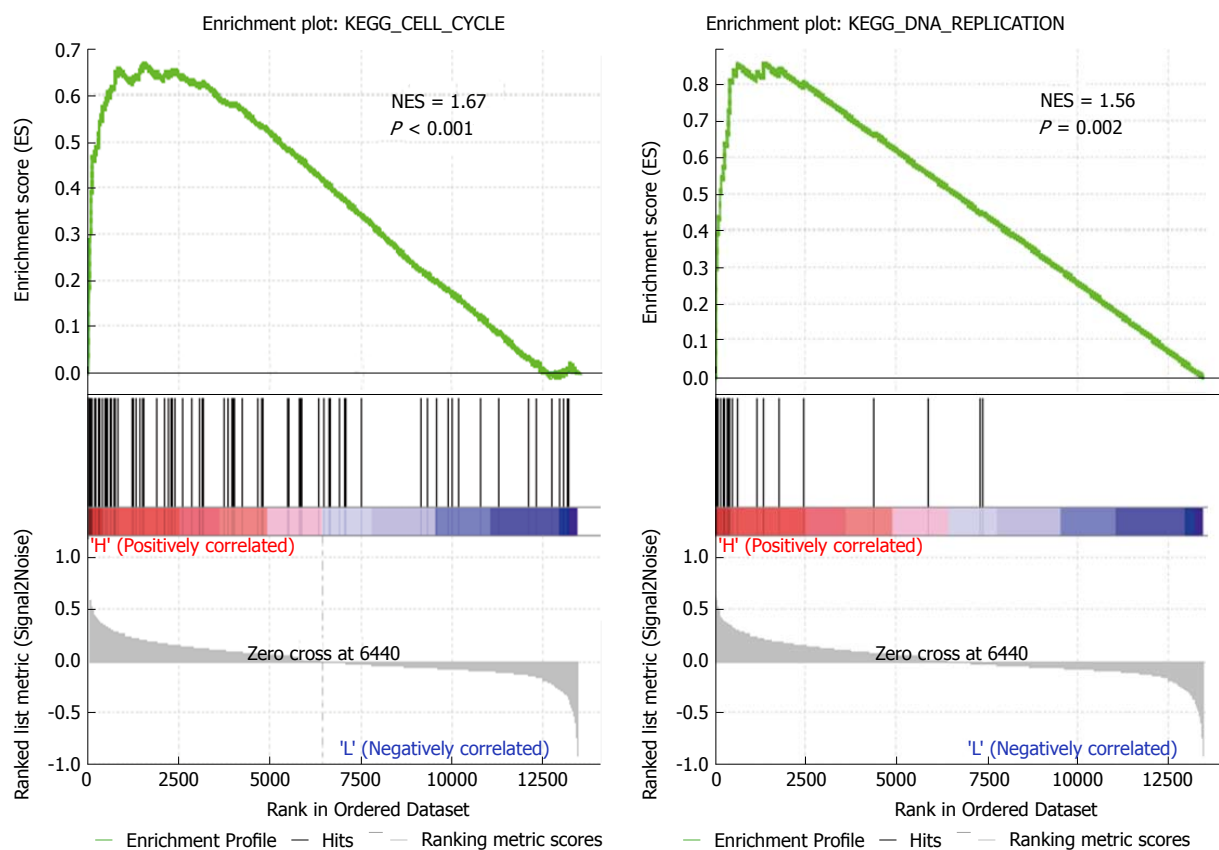
phase (Figure 4C and G). In addition, consistent with the results of GSEA, HMGB3 knockdown also inhibited the expression of cell cycle and DNA replication related genes Cyclin B1, proliferating cell nuclear antigen (PCNA) and flap structure-specific endonuclease 1 (FEN1), indicating that HMGB3 might promote the proliferation of HCC cells by regulating cell cycle and DNA replication pathway (Figure 4D-F).

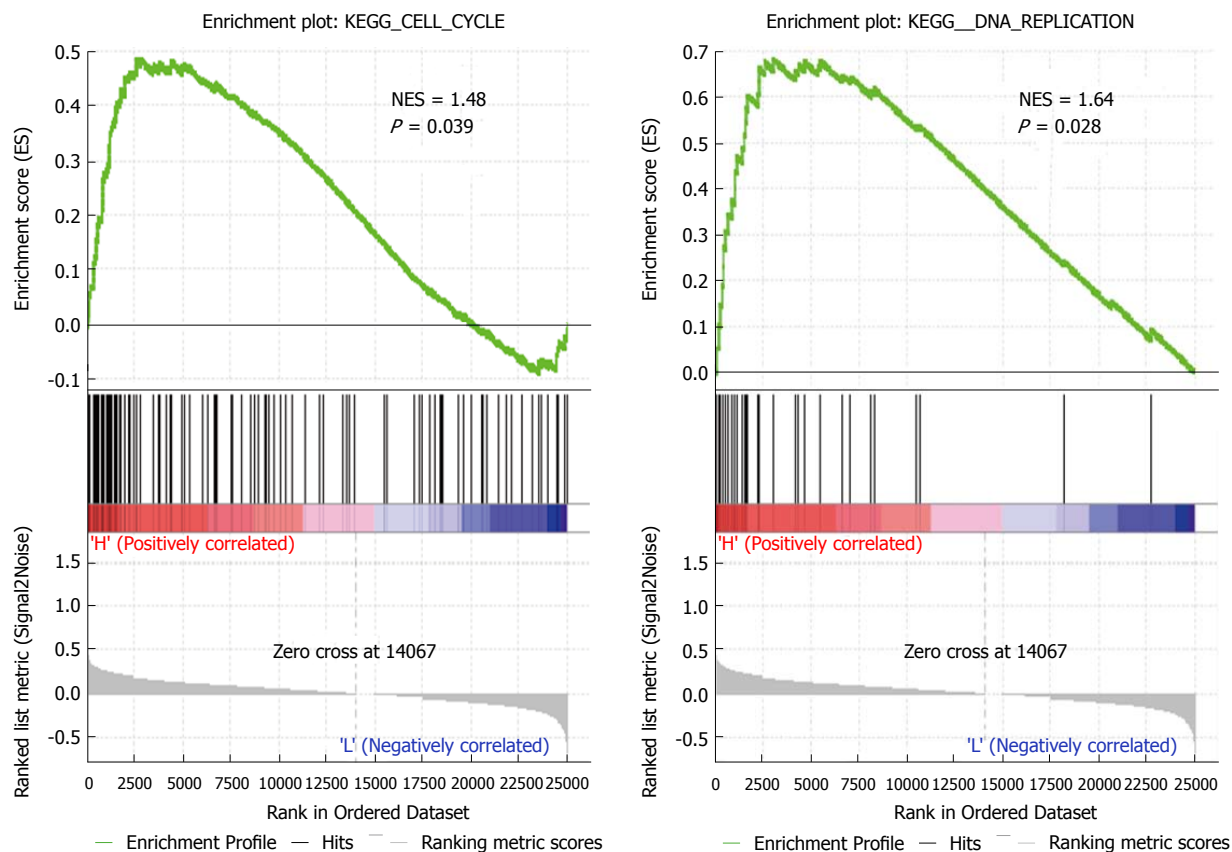**Silencing HMGB3 inhibited tumor growth**

Compared with the NC-shRNA group, significantly decreased tumor volume and weight ($P < 0.001$) was observed in the shRNA-1 group at the 30th d after subcutaneous injection (Figure 5A and C). In addition, silencing HMGB3 by shRNA-1 significantly impeded the growth of xenograft tumors according to the growth curves (Figure 5B). Furthermore, IHC results showed

A

GSE 14520

Enrichment plot: KEGG_CELL_CYCLE

NES = 1.67
P < 0.001

Enrichment plot: KEGG_DNA_REPLICATION

NES = 1.56
P = 0.002

'H' (Positively correlated)

'H' (Positively correlated)

Zero cross at 6440

Zero cross at 6440

'L' (Negatively correlated)

'L' (Negatively correlated)

Rank in Ordered Dataset

Rank in Ordered Dataset

— Enrichment Profile — Hits — Ranking metric scores

— Enrichment Profile — Hits — Ranking metric scores

TCGA

Enrichment plot: KEGG_CELL_CYCLE

NES = 1.48
P = 0.039

Enrichment plot: KEGG__DNA_REPLICATION

NES = 1.64
P = 0.028

'H' (Positively correlated)

'H' (Positively correlated)

Zero cross at 14067

Zero cross at 14067

'L' (Negatively correlated)

'L' (Negatively correlated)

Rank in Ordered Dataset

Rank in Ordered Dataset

— Enrichment Profile — Hits — Ranking metric scores

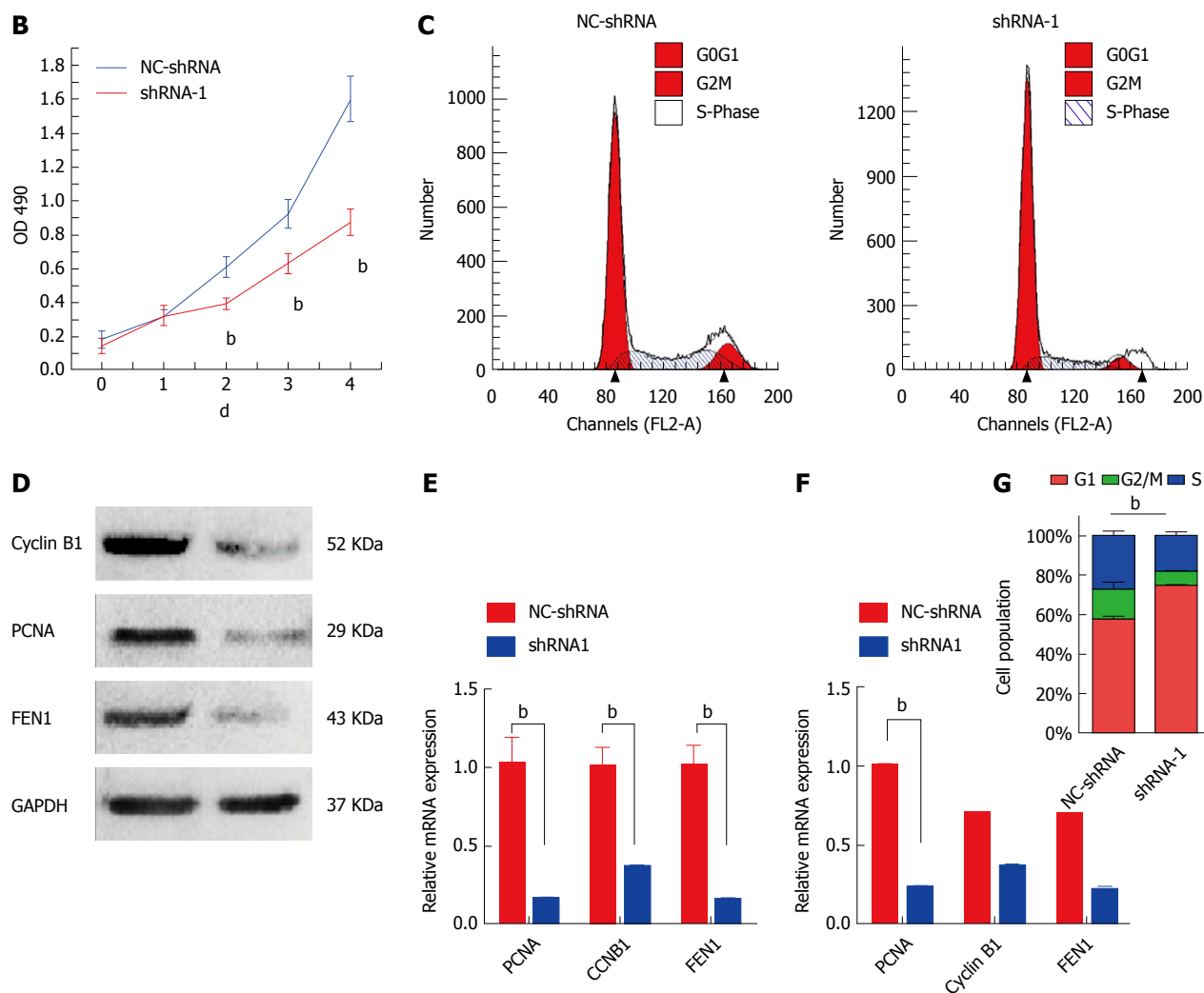— Enrichment Profile — Hits — Ranking metric scores

Figure 4 Knockdown *HMGB3* inhibited cell cycle and proliferation of hepatocellular carcinoma cells. A: Gene set enrichment analysis (GSEA) was conducted to sort the pathways according to HMGB3 expression in GSE14520 and TCGA. Cell cycle and DNA replication pathways were found to be significantly correlated with HMGB3 expression. B: Proliferation of HepG2 cells transfected with shRNA-1 and NC-shRNA was detected using the MTT method. C: Cell cycle of HepG2 cells was analyzed after transfection with shRNA using flow cytometry. D: According to the GSEA analysis, three genes (CCNB1, PCNA, and FEN1) involved in cell cycle and DNA replication were detected after shRNA transfection using RT-qPCR. E: Western blotting was conducted to observe the protein expression of cyclin B1, PCNA, and FEN1 in the NC-shRNA and shRNA-1 group. F: Each bar represents the corresponding intensity in E normalized to GAPDH. G: Percentage columns represent the distribution of the cell cycle in corresponding groups. [b]$P < 0.01$.

that Ki67 expression in tumor tissues of the shRNA-1 group was significantly lower ($P < 0.01$) than that of the NC-shRNA group, indicating that HMGB3 might contribute to the proliferation of HCC cells *in vivo* (Figure 5D).

DISCUSSION

The HMGB family has been recognized as an important regulator in tumor progression[18]. Although the HMGB family with various physiological and pathological functions was previously associated with liver cancer, neither HMGB1 nor HMGB2 was reported to exhibit dynamic expression in tumorigenesis[26]. In this study, the rat hepatocarcinogenesis model was conducted to analyze the expression characteristics from HMGB1, HMGB2, HMGB3, and HMGB4. No statistical differences in HMGB1 or HMGB2 expression were observed. However,

HMGB3 and HMGB4 expression were upregulated and downregulated, respectively through the malignant transformation *in vivo*. To further verify the HMGB3 expression in HCC, several HCC-related bioinformatics databases were assessed. Interestingly, consistent with the results of HCC model, analyses of normalized $\log_2$ transformed microarray expression data sets clearly confirmed the significant upregulation of HMGB3 mRNA in human HCC tissues, and especially in advanced HCC tissues, indicating that HMGB3 might be an oncogenic protein involved in the malignant transformation of hepatocytes.

HMGB proteins can assist in either activating or repressing transcription[27]. In adult vertebrates, HMGB1 is found in all cell types, whereas HMGB3 mRNA was reported to be absent in most adult tissues[7,28]. Indeed, overexpression of HMGB3 has been discovered in several cancer types and correlated with clinical features of cancer patients, including advanced tumor-node-
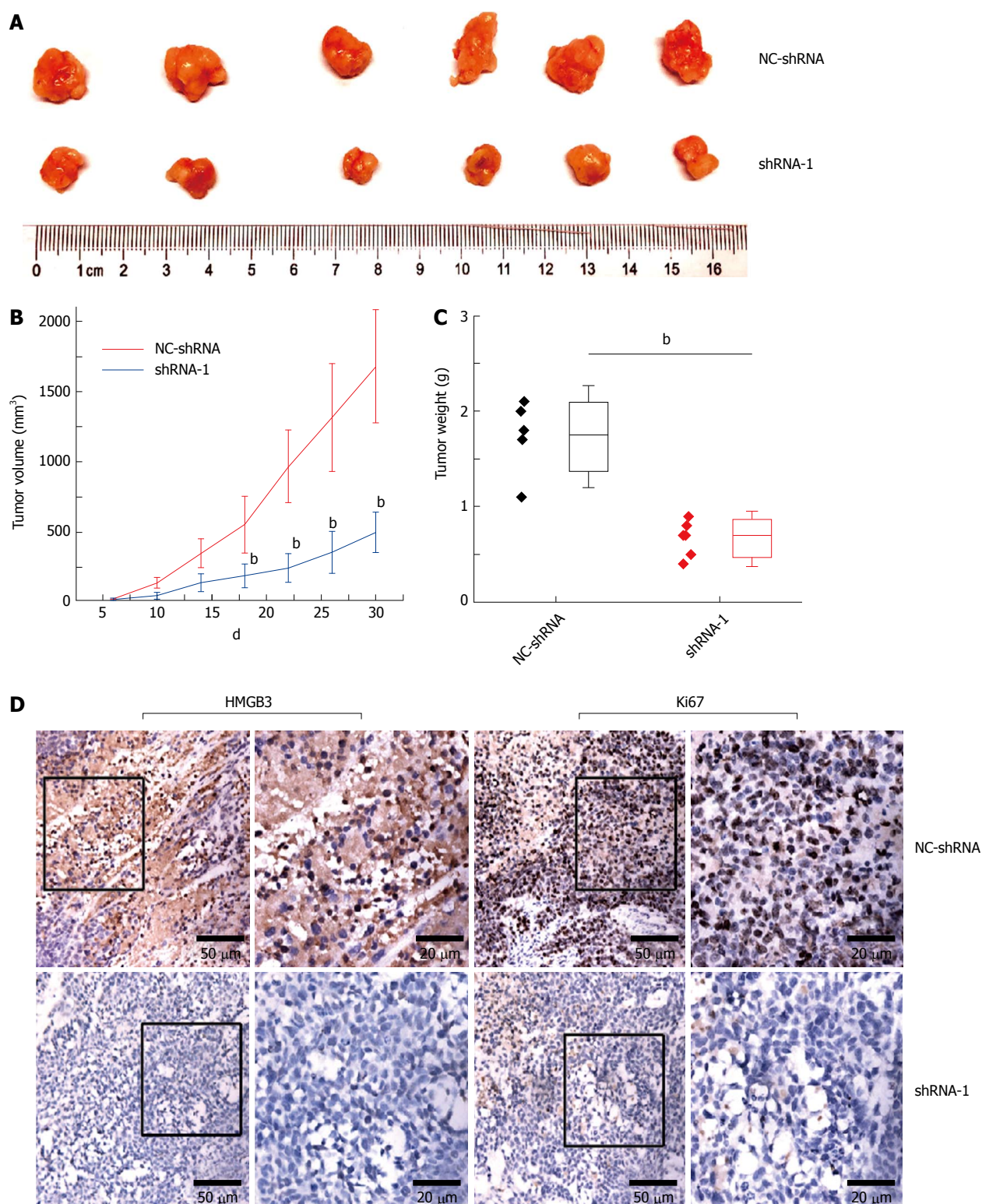
Figure 5 Silencing *HMGB3* suppressed growth of xenograft tumor. HepG2 cells transfected with NC-shRNA and shRNA-1 were subcutaneously injected into mice. A: The morphology of the xenograft tumor in the NC-shRNA or shRNA-1 group at 30th d. B: The growth curves of tumors derived from HepG2 cells in the NC-shRNA or shRNA-1 group. C: The weight of xenograft tumors in the NC-shRNA or shRNA-1 group. D: The immunochemical staining of HMGB3 and Ki67 in xenograft tumor tissues in the NC-shRNA or shRNA-1 group. $^{b}P < 0.01$.

metastasis (TNM) stage, serosal invasion, and overall survival[23,28]. The rat hepatocarcinogenesis model also indicated the potential role of HMGB3 in HCC progression. However, the expression features of HMGB3 and its roles in HCC are still unclear. Thus, the current study

further investigated HMGB3 expression in HCC cell lines. Interestingly, overexpression of HMGB3 was observed in HCC cells rather than normal hepatocytes, which was consistent with the hepatocarcinogenesis model and bioinformatic analysis.

HMGB3 has been reported to play crucial roles in tumor progression by contributing to malignant behaviors and regulating oncogenic pathways. For instance, HMGB3 could enhance the migration and growth of gastric cancer cells *via* activation of the Wnt pathway[23]. It also could increase the proliferation and invasion of breast cancer cells as a target gene of miRNA-205[29]. However, for HCC, the malignant behaviors and mechanism mediated by HMGB3 still remain unclear. Thus, bioinformatic analysis was conducted to explore the underlying roles. Notably, GSEA based on GEO database and TCGA jointly indicated that overexpression of HMGB3 might be associated with cell cycle and DNA replication pathways. As expected, corresponding *in vitro* studies showed that silencing HMGB3 could significantly inhibit proliferation and induce cell cycle arrest in HCC cells. To further confirm potential mechanisms responsible for the anti-proliferation effects, we explored expression of Cyclin B1, PCNA, and FEN1, which were cell cycle and DNA replication related genes highly enriched in high-HMGB3-mediated pathways in GSEA. Consistent with this prediction, knockdown of HMGB3 obviously downregulated the three genes at the mRNA and protein levels, which have been reported to promote tumor progression[30-32]. Collectively, HMGB3 might promote proliferation of HCC cells by regulating cell cycle and DNA replication pathways.

Although HMGB3 has been correlated with proliferation, chemoresistance, and migration of cancer cells in previous studies[23,33,34], as far as we know, there was no xenograft assays to evaluate HMGB3 as a regulator of tumor growth *in vivo*. Given the interesting results from the rat model and *in vitro* study, our current study further evaluated HMGB3 as an important regulator of tumor growth *in vivo*. Xenograft tumors derived from HMGB3-silenced HCC cells grew slower than the control tumors, with an obvious reduction in the proliferation marker Ki67. It suggested that HMGB3 might contribute to the tumor growth *in vivo* via regulating proliferation of HCC cells.

In conclusion, to the best of our knowledge, this is the first report to investigate HMGB3 expression and indicate that it may be a novel diagnostic marker or therapeutic target for HCC. Here, the findings are promising, and the initial evidence confirmed that HMGB3 is one of the key molecules in HCC progression. Future studies should clarify the molecular mechanisms of the upregulation of HMGB3 expression and its important role in hepatocarcinogenesis to elucidate how HMGB3 might promote proliferation of HCC cells and tumor growth by regulating cell cycle and DNA replication pathways.

ARTICLE HIGHLIGHTS

Research background
Hepatocellular carcinoma (HCC) is one of the most common and fatal malignancies worldwide with a multi-factorial, multistep, complex process, and poor prognosis. Early diagnosis of HCC at an early stage is of the utmost importance. This found a new molecular biomarker to monitor the malignant

transformation of hepatocytes.

Research motivation
Although serum alpha fetoprotein (AFP) level is a useful tumor marker for the detection and monitoring of HCC, the false-negative rate with AFP level alone may be as high as 40% for patients with early stage HCC. Even in patients with advanced HCC, the AFP levels may remain normal in 15%-30% of the patients. New specific markers, such as circulating HS-GGT, HS-AFP or AFP-L3, miRNA, GPC-3, and GP73, have been developed to improve the sensitivity, specificity, early detection, and prediction of prognosis. However, the overall results have been unsatisfactory.

Research objectives
The most urgent needs are to find sensitive markers for early diagnosis or monitor postoperative recurrence, and to give adequate treatment for HCC. It has many characteristics, such as fast infiltrating growth, metastasis in early stage, high-grade malignancy, and poorly therapeutic efficacy, thus the prognosis is poor and early detection is of the utmost importance. The present study focused on exploring the relationship between dynamic expression of HMGB-3 and malignant transformation of hepatocytes.

Research methods
Dynamic models of rat hepatocarcinogenesis were made to investigate the expression of the high mobility group box (HMGB) family. HMGB3 expression was measured at the protein level by immunohistochemistry or Western blotting and at the mRNA level by real time PCR. Human HMGB3 expression was evaluated using bioinformatics databases and its mechanisms were analyzed *in vitro*. Xenograft growth was also measured.

Research results
HMGB3 expression was upregulated through the malignant transformation of liver cells *in vivo*.

Research conclusions
The upregulation of liver HMGB3 expression was found by dynamic model of hepatocytes malignant transformation with the alterations of rat liver histopathology. This was confirmed by mining HMGB3 expression in human HCC tissues in bioinformatic databases. Further studies elucidating the role HMGB3 plays in regulating HCC progression, suggests that HMGB3 could be a novel marker for early diagnosis or a molecular therapy target.

Research perspectives
HMGB3 has been confirmed as one of the key molecules in the HMGB family with HCC development. However, the molecular mechanisms of the upregulation of HMGB3 expression and its important role in hepatocarcinogenesis should be clarified in promoting proliferation of HCC cells and tumor growth and regulating cell cycle and DNA replication pathways in the future.

ACKNOWLEDGMENTS

REFERENCES

1   **Forner A**, Reig M, Bruix J. Hepatocellular carcinoma. *Lancet* 2018; **391**: 1301-1314 [PMID: 29307467 DOI: 10.1016/S0140-6736(18)30010-2]
2   **Ryerson AB**, Eheman CR, Altekruse SF, Ward JW, Jemal A, Sherman RL, Henley SJ, Holtzman D, Lake A, Noone AM, Anderson RN, Ma J, Ly KN, Cronin KA, Penberthy L, Kohler BA. Annual Report to the Nation on the Status of Cancer, 1975-2012, featuring the increasing incidence of liver cancer. *Cancer* 2016; **122**: 1312-1337 [PMID: 26959385 DOI: 10.1002/cncr.29936]

3   **Yi SW**, Choi JS, Yi JJ, Lee YH, Han KJ. Risk factors for hepatocellular carcinoma by age, sex, and liver disorder status: A prospective cohort study in Korea. *Cancer* 2018; **124**: 2748-2757 [PMID: 29669170 DOI: 10.1002/cncr.31406]

4   **Eso Y**, Marusawa H. Novel approaches for molecular targeted therapy against hepatocellular carcinoma. *Hepatol Res* 2018; **48**: 597-607 [PMID: 29689631 DOI: 10.1111/hepr.13181]

5   **Trojan J**, Waidmann O. Role of regorafenib as second-line therapy and landscape of investigational treatment options in advanced hepatocellular carcinoma. *J Hepatocell Carcinoma* 2016; **3**: 31-36 [PMID: 27703962 DOI: 10.2147/JHC.S112537]

6   **Llovet JM**, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J; SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378-390 [PMID: 18650514 DOI: 10.1056/NEJMoa0708857]

7   **Reeves R**. High mobility group (HMG) proteins: Modulators of chromatin structure and DNA repair in mammalian cells. *DNA Repair (Amst)* 2015; **36**: 122-136 [PMID: 26411874 DOI: 10.1016/j.dnarep.2015.09.015]

8   **Fedele M**, Fusco A. HMGA and cancer. *Biochim Biophys Acta* 2010; **1799**: 48-54 [PMID: 20123067 DOI: 10.1016/j.bbagrm.2009.11.007]

9   **Stros M**. HMGB proteins: interactions with DNA and chromatin. *Biochim Biophys Acta* 2010; **1799**: 101-113 [PMID: 20123072 DOI: 10.1016/j.bbagrm.2009.09.008]

10   **Gerlitz G**. HMGNs, DNA repair and cancer. *Biochim Biophys Acta* 2010; **1799**: 80-85 [PMID: 20004154 DOI: 10.1016/j.bbagrm.2009.10.007]

11   **Kajino K**, Yamamoto T, Hayashi J, Umeda T, Takahara T, Hino O. Recombination hot spot of hepatitis B virus genome binds to members of the HMG domain protein family and the Y box binding protein family; implication of these proteins in genomic instability. *Intervirology* 2001; **44**: 311-316 [PMID: 11684893 DOI: 10.1159/000050063]

12   **Tohme S**, Yazdani HO, Liu Y, Loughran P, van der Windt DJ, Huang H, Simmons RL, Shiva S, Tai S, Tsung A. Hypoxia mediates mitochondrial biogenesis in hepatocellular carcinoma to promote tumor growth through HMGB1 and TLR9 interaction. *Hepatology* 2017; **66**: 182-197 [PMID: 28370295 DOI: 10.1002/hep.29184]

13   **Chen S**, Dong Z, Yang P, Wang X, Jin G, Yu H, Chen L, Li L, Tang L, Bai S, Yan H, Shen F, Cong W, Wen W, Wang H. Hepatitis B virus X protein stimulates high mobility group box 1 secretion and enhances hepatocellular carcinoma metastasis. *Cancer Lett* 2017; **394**: 22-32 [PMID: 28216372 DOI: 10.1016/j.canlet.2017.02.011]

14   **Zhang D**, Cao J, Zhong Q, Zeng L, Cai C, Lei L, Zhang W, Liu F. Long noncoding RNA PCAT-1 promotes invasion and metastasis via the miR-129-5p-HMGB1 signaling pathway in hepatocellular carcinoma. *Biomed Pharmacother* 2017; **95**: 1187-1193 [PMID: 28931210 DOI: 10.1016/j.biopha.2017.09.045]

15   **Lv G**, Wu M, Wang M, Jiang X, Du J, Zhang K, Li D, Ma N, Peng Y, Wang L, Zhou L, Zhao W, Jiao Y, Gao X, Hu Z. miR-320a regulates high mobility group box 1 expression and inhibits invasion and metastasis in hepatocellular carcinoma. *Liver Int* 2017; **37**: 1354-1364 [PMID: 28317284 DOI: 10.1111/liv.13424]

16   **Lu L**, Qiu C, Li D, Bai G, Liang J, Yang Q. MicroRNA-505 suppresses proliferation and invasion in hepatoma cells by directly targeting high-mobility group box 1. *Life Sci* 2016; **157**: 12-18 [PMID: 27259809 DOI: 10.1016/j.lfs.2016.05.039]

17   **Kwon JH**, Kim J, Park JY, Hong SM, Park CW, Hong SJ, Park SY, Choi YJ, Do IG, Joh JW, Kim DS, Choi KY. Overexpression of high-mobility group box 2 is associated with tumor aggressiveness and prognosis of hepatocellular carcinoma. *Clin Cancer Res* 2010; **16**: 5511-5521 [PMID: 20851854 DOI: 10.1158/1078-0432.CCR-10-0825]

18   **Ueda T**, Yoshida M. HMGB proteins and transcriptional regulation. *Biochim Biophys Acta* 2010; **1799**: 114-118 [PMID: 20123073 DOI: 10.1016/j.bbagrm.2009.11.005]

19   **Gong Y**, Cao Y, Song L, Zhou J, Wang C, Wu B. HMGB3 characterization in gastric cancer. *Genet Mol Res* 2013; **12**: 6032-6039 [PMID: 24338397 DOI: 10.4238/2013.December.2.1]

20   **Song N**, Liu B, Wu JL, Zhang RF, Duan L, He WS, Zhang CM. Prognostic value of HMGB3 expression in patients with non-small cell lung cancer. *Tumour Biol* 2013; **34**: 2599-2603 [PMID: 23609034 DOI: 10.1007/s13277-013-0807-y]

21   **Gao J**, Zou Z, Gao J, Zhang H, Lin Z, Zhang Y, Luo X, Liu C, Xie J, Cai C. Increased expression of HMGB3: a novel independent prognostic marker of worse outcome in patients with esophageal squamous cell carcinoma. *Int J Clin Exp Pathol* 2015; **8**: 345-352 [PMID: 25755721]

22   **Li X**, Wu Y, Liu A, Tang X. MiR-27b is epigenetically downregulated in tamoxifen resistant breast cancer cells due to promoter methylation and regulates tamoxifen sensitivity by targeting HMGB3. *Biochem Biophys Res Commun* 2016; **477**: 768-773 [PMID: 27363334 DOI: 10.1016/j.bbrc.2016.06.133]

23   **Zhang Z**, Chang Y, Zhang J, Lu Y, Zheng L, Hu Y, Zhang F, Li X, Zhang W, Li X. HMGB3 promotes growth and migration in colorectal cancer by regulating WNT/β-catenin pathway. *PLoS One* 2017; **12**: e0179741 [PMID: 28678825 DOI: 10.1371/journal.pone.0179741]

24   **Li M**, Cai Y, Zhao H, Xu Z, Sun Q, Luo M, Gu L, Meng M, Han X, Sun H. Overexpression of HMGB3 protein promotes cell proliferation, migration and is associated with poor prognosis in urinary bladder cancer patients. *Tumour Biol* 2015; **36**: 4785-4792 [PMID: 25647262 DOI: 10.1007/s13277-015-3130-y]

25   **Yan XD**, Yao M, Wang L, Zhang HJ, Yan MJ, Gu X, Shi Y, Chen J, Dong ZZ, Yao DF. Overexpression of insulin-like growth factor-I receptor as a pertinent biomarker for hepatocytes malignant transformation. *World J Gastroenterol* 2013; **19**: 6084-6092 [PMID: 24106410 DOI: 10.3748/wjg.v19.i36.6084]

26   **Afrin R**, Arumugam S, Rahman A, Wahed MI, Karuppagounder V, Harima M, Suzuki H, Miyashita S, Suzuki K, Yoneyama H, Ueno K, Watanabe K. Curcumin ameliorates liver damage and progression of NASH in NASH-HCC mouse model possibly by modulating HMGB1-NF-κB translocation. *Int Immunopharmacol* 2017; **44**: 174-182 [PMID: 28110063 DOI: 10.1016/j.intimp.2017.01.016]

27   **Yanai H**, Ban T, Taniguchi T. High-mobility group box family of proteins: ligand and sensor for innate immunity. *Trends Immunol* 2012; **33**: 633-640 [PMID: 23116548 DOI: 10.1016/j.it.2012.10.005]

28   **Tang HR**, Luo XQ, Xu G, Wang Y, Feng ZJ, Xu H, Shi YW, Zhang Q, Wu LG, Xue CQ, Wang CW, Wu CY. High mobility group-box 3 overexpression is associated with poor prognosis of resected gastric adenocarcinoma. *World J Gastroenterol* 2012; **18**: 7319-7326 [PMID: 23326140 DOI: 10.3748/wjg.v18.i48.7319]

29   **Elgamal OA**, Park JK, Gusev Y, Azevedo-Pouly AC, Jiang J, Roopra A, Schmittgen TD. Tumor suppressive function of mir-205 in breast cancer is linked to HMGB3 regulation. *PLoS One* 2013; **8**: e76402 [PMID: 24098490 DOI: 10.1371/journal.pone.0076402]

30   **Huang V**, Place RF, Portnoy V, Wang J, Qi Z, Jia Z, Yu A, Shuman M, Yu J, Li LC. Upregulation of Cyclin B1 by miRNA and its implications in cancer. *Nucleic Acids Res* 2012; **40**: 1695-1707 [PMID: 22053081 DOI: 10.1093/nar/gkr934]

31   **Juríková M**, Danihel Ľ, Polák Š, Varga I. Ki67, PCNA, and MCM proteins: Markers of proliferation in the diagnosis of breast cancer. *Acta Histochem* 2016; **118**: 544-552 [PMID: 27246286 DOI: 10.1016/j.acthis.2016.05.002]

32   **He L**, Luo L, Zhu H, Yang H, Zhang Y, Wu H, Sun H, Jiang F, Kathera CS, Liu L, Zhuang Z, Chen H, Pan F, Hu Z, Zhang J, Guo Z. FEN1 promotes tumor progression and confers cisplatin resistance in non-small-cell lung cancer. *Mol Oncol* 2017; **11**: 640-654 [PMID: 28371273 DOI: 10.1002/1878-0261.12058]

33   **Guo S**, Wang Y, Gao Y, Zhang Y, Chen M, Xu M, Hu L, Jing Y, Jing F, Li C, Wang Q, Zhu Z. Knockdown of High Mobility Group-Box 3 (HMGB3) Expression Inhibits Proliferation, Reduces

Migration, and Affects Chemosensitivity in Gastric Cancer Cells. *Med Sci Monit* 2016; **22**: 3951-3960 [PMID: 27774979 DOI: 10.12659/MSM.900880]

34   **Yamada Y**, Nishikawa R, Kato M, Okato A, Arai T, Kojima S, Yamazaki K, Naya Y, Ichikawa T, Seki N. Regulation of HMGB3 by antitumor miR-205-5p inhibits cancer cell aggressiveness and is involved in prostate cancer pathogenesis. *J Hum Genet* 2018; **63**: 195-205 [PMID: 29196733 DOI: 10.1038/s10038-017-0371-1]

Retrospective Cohort Study

C-peptide as a key risk factor for non-alcoholic fatty liver disease in the United States population

Amporn Atsawarungruangkit, Jirat Chenbhanich, George Dickstein

Amporn Atsawarungruangkit, Jirat Chenbhanich, George Dickstein, Department of Medicine, MetroWest Medical Center, Framingham, MA 01702, United States

Amporn Atsawarungruangkit, Jirat Chenbhanich, George Dickstein, Department of Medicine, Tufts University School of Medicine, Boston, MA 02111, United States

ORCID number: Amporn Atsawarungruangkit (0000-0003-0622-6839); Jirat Chenbhanich (0000-0002-2274-5965); George Dickstein (0000-0003-0100-5498).

Author contributions: Atsawarungruangkit A contributed to the study concept and design, analysis and interpretation of data, and preparation of manuscript; Chenbhanich J and Dickstein G contributed to interpretation of data and preparation and manuscript; all authors read and approved the final manuscript.

Institutional review board statement: This study uses the publicly available data from the Third National Health and Nutrition Examination Survey (NHANES III), which is conducted by the National Center for Health Statistics (NCHS). The NHANES protocol was approved by the NCHS Research Ethics Review Board.

Informed consent statement: In NHANES Ⅲ, the consent form was signed by participants in the survey.

Conflict-of-interest statement: No conflict of interest exists.

Manuscript source: Unsolicited manuscript

Correspondence to: Amporn Atsawarungruangkit, BPharm, MD, Doctor, Research Fellow, Department of Medicine, MetroWest Medical Center, 115 Lincoln St., Framingham, MA 01702, United States. a.atsawarungraungkit.@mwmc.com
Telephone: +1-857-3126114

Abstract

AIM
To determine whether fasting C-peptide is an independent predictor for non-alcoholic fatty liver disease (NAFLD) in United States population.

METHODS
Using the National Health and Nutrition Examination Survey (NHANES) 1988-1994, NAFLD participants aged 20 or greater without any other liver diseases were included in this study. Excessive alcohol intake is defined as > 2 drinks per day for males and > 1 drink per day for females. C-peptide and 27 other factors known to be associated with NAFLD (*e.g.*, age, gender, body mass index, waist circumference, race/ethnicity, liver chemistries, and other diabetes tests) were tested in both univariate and multivariate level using logistic regression with a *P*-value 0.05.

RESULTS
Of 18825 participants aged ⩾ 20, 3235 participants (*n* = 3235) met inclusion criteria. There were 23 factors associated with NAFLD by univariate analysis. 9 factors, ranked by the highest change in pseudo R^2, were found to be significant predictors of NAFLD in multivariate model: waist circumference, fasting C-peptide, natural log of alanine aminotransferase (ALT), total protein, being

Mexican American, natural log of glycated hemoglobin, triglyceride level, being non-Hispanic white, and ferritin level.

CONCLUSION

Together with waist circumference and ALT, fasting C-peptide is among three most important predictors of NAFLD in United States population in the NHANES data set. Further study is needed to validate the clinical utility of fasting C-peptide in diagnosis or monitoring insulin resistance in NAFLD patients.

Key words: Insulin resistance; Fatty liver; Hepatosteatosis; Metabolic syndrome; C-peptide

Core tip: Non-alcoholic fatty liver disease (NAFLD) is a growing global epidemic and associated with many conditions and factors, including insulin resistance. However, C-peptide has not been used in practice to assess insulin resistance in NAFLD patients. Using a large national dataset, we demonstrated that three most important risk factors for NAFLD are waist circumference, fasting C-peptide, and alanine aminotransferase, respectively. Such results revealed that C-peptide superior to measurement of fasting insulin levels and can potentially be used for screening or monitoring the degree of insulin resistance in NAFLD.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a condition in which hepatic steatosis exists in the absence of excessive alcohol consumption. NAFLD is the most common cause of chronic liver disease in the United States with estimated prevalence around 30%-40%[1-3]. Given the epidemic of obesity, NAFLD is increasingly prevalent and challenging[4,5]. NAFLD can progress to more severe liver diseases, such as non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma.

Obesity and insulin resistance are among important risk factors for NAFLD[6,7]. Many studies found that indicators of obesity [*i.e.*, body mass index (BMI) and waist circumference] and insulin resistance [*i.e.*, glycated hemoglobin (HbA1c), insulin level, fasting glucose, and diabetes mellitus] are independently associated with NAFLD and/or severity of liver fibrosis in NAFLD[8-11]. C-peptide levels can be used to measure insulin secretion[12]. However, there is limited evidence

of the association between NAFLD and C-peptide at the multivariate level[13,14].

Both C-peptide and insulin are produced and released in equimolar amounts. C-peptide can therefore be used to assess endogenous insulin secretion. However, the level of C-peptide and insulin level in blood are typically different deriving from the differences in clearance mechanisms and half-life[15]. In addition to diabetes and insulin resistance, C-peptide has been associated with many risk factors for NAFLD including cardiovascular diseases and metabolic syndrome[16-18].

Therefore, our primary objective was to determine if fasting C-peptide is independently associated with NAFLD using multivariate analysis in the United States general population.

MATERIALS AND METHODS

Study population and study design

The Third National Health and Nutrition Examination Survey (NHANES Ⅲ) is a probability sample of 39695 persons aged 2 mo and older representing the United States population and conducted by the National Center for Health Statistics (NCHS) to evaluate health and nutritional status[19]. The survey collected multiple data sets, including demographic, interviews, physical examinations, and laboratory testing of biologic samples. NHANES Ⅲ was conducted from 1988 to 1994. The NHANES protocol was approved by the NCHS Research Ethics Review Board.

There were 18825 persons aged 20 years or older in NHANES Ⅲ that met inclusion criteria for this study. The exclusion criteria included: (1) Ungradable or missing ultrasound results for hepatic steatosis, (2) excessive alcohol consumption, (3) hepatitis B or hepatitis C infection (4) fasting period outside of 8-24 h (5) incomplete or missing data on physical examination and laboratory testing. Participants were divided into two groups: NAFLD participants (study group) and non-NAFLD participants (control group).

As presented in Table 1, we included 28 factors associated with NAFLD as independent variables in this study: Demographic (*i.e.*, age , gender, race/ethnicity), body measurement (*i.e.*, BMI and waist circumference), general biochemistry tests [*i.e.*, iron, total iron-binding capacity (TIBC), transferrin saturation, ferritin, cholesterol, triglyceride, HDL cholesterol, C-reactive protein, and uric acid], liver chemistry [aspartate aminotransferase (AST), Alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), total bilirubin, total protein, and albumin], and diabetes testing profile [*i.e.*, HbA1c, fasting plasma glucose (FPG), fasting C-peptide, and fasting insulin]. Besides demographic variables, the above variables were selected as the risk factors based on the usage in clinical practice and the supporting evidence that demonstrated the association with NAFLD or its commonly accepted risk factors (*i.e.*, obesity, insulin resistance, and liver fibrosis).

Table 1 Baseline characteristics of participants with non-alcoholic fatty liver disease and controls *n* (%)

	NAFLD, *n* = 817	Controls, *n* = 2418
Demographic		
Age (yr)	48.47 ± 15.75	44.36 ± 16.20
Gender (male)	368 (45.04)	1004 (41.52)
Race/ethnicity		
White (non-Hispanic)	308 (37.70)	1046 (43.13)
Black (non-Hispanic)	193 (23.62)	705 (29.16)
Mexican American	280 (34.27)	550 (22.75)
Others	36 (4.41)	120 (4.96)
Body measurement		
Body mass index (kg/m^2)	30.38 ± 6.95	26.56 ± 5.42
Waist circumference (cm)	101.73 ± 16.32	90.84 ± 13.45
Biochemistry tests		
Iron (μg/dL)	75.35 ± 29.71	77.71 ± 32.75
TIBC (μg/dL)	364.79 ± 58.05	359.86 ± 56.59
Transferrin saturation (%)	21.13 ± 8.73	22.09 ± 9.65
Ferritin (ng/mL)	161.75 ± 152.55	110.16 ± 114.71
Cholesterol (mg/dL)	212.23 ± 44.95	202.73 ± 42.19
Triglyceride (mg/dL)	202.91 ± 137.97	136.58 ± 95.79
HDL cholesterol (mg/dL)	46.72 ± 16.80	52.10 ± 15.61
C-reactive protein (mg/dL)[1]	0.56 ± 0.80	0.45 ± 0.65
Uric acid (mg/dL)	5.62 ± 1.52	5.04 ± 1.42
Liver chemistry		
AST (U/L)[1]	24.76 ± 19.62	20.72 ± 14.71
ALT(U/L)[1]	22.78 ± 17.86	15.96 ± 12.14
GGT (U/L)[1]	42.87 ± 66.68	28.14 ± 41.69
ALP (U/L)[1]	93.72 ± 33.61	86.04 ± 36.29
Total bilirubin (mg/dL)	0.55 ± 0.30	0.54 ± 0.28
Total protein (g/dL)	7.49 ± 0.46	7.37 ± 0.45
Albumin (g/dL)	4.11 ± 0.35	4.12 ± 0.36
Diabetes testing profile		
HbA1c (%)[1]	6.02 ± 1.62	5.50 ± 1.09
FPG (mg/dL)[1]	114.50 ± 65.84	97.85 ± 35.68
Fasting C-peptide (pmol/mL)	1.11 ± 0.68	0.69 ± 0.53
Fasting insulin (μU/mL)[1]	21.85 ± 27.82	12.76 ± 19.24

[1]The distribution is positively skewed with skewness > 3. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; FPG: Fasting plasma glucose; GGT: Gamma glutamyl transferase; HbA1c: Glycated hemoglobin; TIBC: Total iron-binding capacity.

Definitions

In this study, NAFLD is defined as: (1) Diagnosed with moderate to severe hepatic steatosis on ultrasound; (2) no history of excessive alcohol intake in the past 12 mo; (3) not infected with hepatitis B or hepatitis C.

To evaluate the presence and extent of hepatic steatosis, readers used five main criteria: (1) Parenchymal brightness, (2) liver to kidney contrast, (3) deep beam attenuation, (4) bright vessel walls, and (5) gallbladder wall definition. Based on the presence or absence of these five criteria, a main finding was categorized as normal, mild, moderate or severe[20]. It is worth nothing that participants aged above 74 were not eligible for ultrasound study in NHANES Ⅲ For this reason, patients age above 74 were excluded from this study.

Excessive alcohol intake is defined as more than 2 drinks per day for men or 1 drink per day for women in the past 12 mo, in which one drink of alcoholic beverage is equivalent to a 12 oz beer, a 5 oz glass of wine, or 1.5 oz of liquor. The average number of drinks per day is calculated from number of drinking days × number of drinks on drinking day/365 d. To qualify as hepatitis viral infection, participants must have tested positive for serum hepatitis B surface antigen or serum hepatitis C antibody HCP (anti-HCV).

Statistical analysis

Statistical analyses were performed using STATA Release 14 (StataCorp LP, TX, United States). Numbers are presented in mean ± SD or number (%). All continuous factors were first tested for skewness; if the distributions were extremely skewed to the right (herein defined as skewness > 3), the factors were log transformed before using them as predictors in regression models. Since the response variable is dichotomous variable (NAFLD or non-NAFLD), logistic model is an appropriate model for determining if predictors are significantly associated with the response variable. As a result, logistic regression was used to determine if NAFLD is associated with any predictor in univariate level. Then, the significant factors from univariate analysis were included as predictors in step-wise logistic regression to determine the significant predictors in multivariate level. The significance level is 0.05.

RESULTS

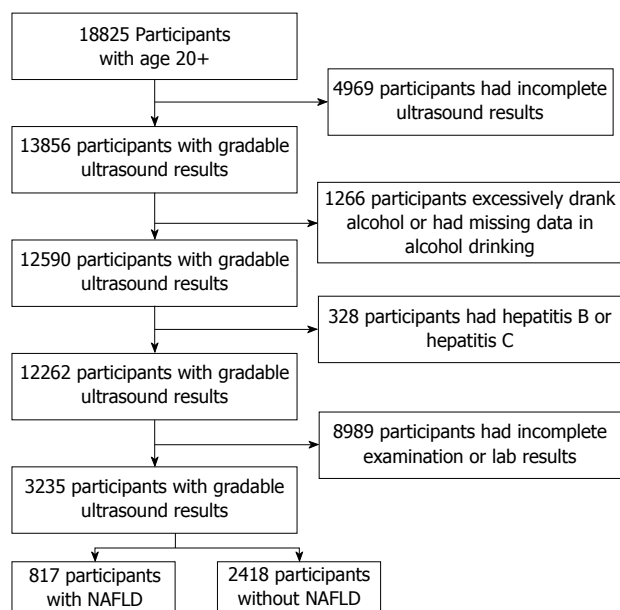Out of 18825 participants aged ≥ 20, there were 3235

Figure 1 Study design and study population.

participants (*n* = 3235) that passed the exclusion criteria as shown in Figure 1. Based on ultrasound findings, 817 (25.26%) participants were classified as NAFLD. Baseline characteristics of participants in study group and control group are summarized in Table 1.

For continuous variables, there were 8 factors having skewness greater than 3. Subsequently, the log transformation was applied to these factors, including C-reactive protein, AST, ALT, GGT, alkaline phosphatase, glycated hemoglobin, plasma glucose, and insulin. As shown in Table 2, there are 24 variables significantly associated with NAFLD in univariate level; the *P*-value of these significant factors mostly below 0.001.

As presented in Table 3, the number of significant factors reduced from 24 to 9 in multivariate analysis. The top three factors ranked by the highest change in pseudo R^2 ($\varDelta R^2$) are waist circumference (OR = 1.03, $\varDelta R^2$ = 2.13%, *P* < 0.001), C-peptide level (OR = 1.82, $\varDelta R^2$ = 1.33%, *P* < 0.001), and $\log_e$ of ALT (OR = 1.76, $\varDelta R^2$ = 1.16%, *P* < 0.001). The pseudo R^2 of the multivariate model is 16.68%.

DISCUSSION

The most significant NAFLD risk factor in both univariate and multivariate levels is waist circumference. Since waist circumference and BMI are highly interrelated surrogate markers of obesity[21], it is not surprising to see one factor eliminated in multivariate level. Waist circumference-a measure of excess abdominal adiposity-has been identified as an independent risk factor for many obesity-related conditions, such as cardiovascular disease, type 2 diabetes, dyslipidemia, and hypertension, metabolic syndrome, polycystic ovary syndrome[22-27]. The results from this study support that waist circumference is also an independent and probably the most important risk

factor for NAFLD in the United States population.

Insulin resistance is another well-known condition commonly found in NAFLD patients[28,29]. Indeed, all diabetes test profiles were positively correlated with NAFLD by univariate analysis. In fact, type 2 diabetes can be diagnosed directly from HbA1c level ($\geqslant$ 6.5%) or FPG ($\geqslant$ 126 mg/dL)[30], while C-peptide and insulin are not routinely used in clinical practice to diagnose type 2 diabetes. While there are situations where C-peptide or insulin levels are useful-the diagnosis of insulinoma[31], surreptitious use of insulin[32], the diagnosis of type 2 diabetes in the young[33], and the diagnosis of latent autoimmune diabetes in adults[34], direct measurement of insulin levels and not of C-peptide has been used to assess insulin resistance. Previous studies often found that C-peptide levels are raised in patients with NASH[35-37]. However, the application of C-peptide as a biomarker for interventions designed to improve insulin sensitivity remains to be determined. In case of NAFLD, there is only limited evidence; C-peptide was found to be associated with NAFLD in specific groups of population (i.e., obese adolescents and adults, latent autoimmune diabetes, and diabetes patients)[13,14,38]. Our results are the first to show that C-peptide has a role not only as an independent risk factor for NAFLD but can also be useful for screening or monitoring the degree of insulin resistance in NAFLD in the general population. Based on $\varDelta R^2$, we conclude that insulin resistance, as indicated by fasting C-peptide, is the second most important condition leading to NAFLD, second only to obesity as diagnosed by waist circumference, and is superior to measurement of fasting insulin levels.

Liver chemistries are used as an indicator of liver inflammation or liver cell damage. Commonly used liver chemistries include AST, ALT, ALP, GGT, total bilirubin, total protein, and albumin. For example, predominance of AST and ALT indicates hepatocellular injury; predominance of ALP and total bilirubin indicates cholestatic injury; an elevated ALP of hepatic origin may be confirmed GGT[39-41]. As shown in Table 2, total protein and the natural log of AST, ALT, ALP, and GGT were positively associated with NAFLD in univariate analysis. However, only total protein and natural log of ALT were positively correlated with NAFLD in multivariate level. AST and ALT are the most widely used liver chemistries. The fact that ALT is included in multivariate model is not unexpected since ALT is generally higher than AST level in NAFLD[40]. On the other hand, total protein is a non-specific marker of health, nutrition and liver synthetic capacity. Due to the fact that total protein consists of albumin and multiple subtypes of globulin, further investigation into the association between NAFLD and each subtype of globulin may provide a clearer explanation of our findings.

Ferritin is a protein that mainly stores iron in the body and serum ferritin level is the most accurate blood test to diagnose iron deficiency anemia[42]. Recently, the role of ferritin as a biomarker in inflammatory diseases has been increasingly recognized[43-45]. As an acute phase

Table 2 Univariate analysis for predictors of non-alcoholic fatty liver disease

	Beta	Standard error	Odds ratio	*P* value
Demographic				
Age (yr)	0.0157	0.0025	1.02	< 0.001[a]
Gender (male)	0.1435	0.0815	1.15	0.078
Race/ethnicity				
White (non-Hispanic)	-0.226	0.0831	0.80	0.007[a]
Black (non-Hispanic)	-0.2857	0.0937	0.75	0.002[a]
Mexican American	0.5715	0.0882	1.77	< 0.001[a]
Others	-0.1248	0.1945	0.88	0.521
Body measurement				
Body mass index (kg/m^2)	0.1004	0.0069	1.11	< 0.001[a]
Waist circumference (cm)	0.0506	0.003	1.05	< 0.001[a]
Biochemistry tests				
Iron (μg/dL)	-0.0024	0.0013	1.00	0.069
TIBC (μg/dL)	0.0015	0.0007	1.00	0.032[a]
Transferrin saturation (%)	-0.0111	0.0044	0.99	0.012[a]
Ferritin (ng/mL)	0.0029	0.0003	1.00	< 0.001[a]
Cholesterol (mg/dL)	0.005	0.0009	1.01	< 0.001[a]
Triglyceride (mg/dL)	0.0049	0.0004	1.00	< 0.001[a]
HDL cholesterol (mg/dL)	-0.0261	0.0031	0.97	< 0.001[a]
C-reactive protein (mg/dL)[1]	0.3398	0.0534	1.40	< 0.001[a]
Uric acid (mg/dL)	0.2649	0.0277	1.30	< 0.001[a]
Liver chemistry				
AST (U/L)[1]	1.004	0.1153	1.02	< 0.001[a]
ALT (U/L)[1]	1.0274	0.0777	1.04	< 0.001[a]
GGT (U/L)[1]	0.7441	0.0612	1.01	< 0.001[a]
ALP (U/L)[1]	0.9011	0.1303	1.01	< 0.001[a]
Total bilirubin (mg/dL)	0.0914	0.1395	1.09	0.512
Total protein (g/dL)	0.581	0.0896	1.79	< 0.001[a]
Albumin (g/dL)	-0.0871	0.1141	0.92	0.445
Diabetes testing profile				
HbA1c (%)[1]	2.2201	0.2184	1.34	< 0.001[a]
FPG (mg/dL)[1]	1.3125	0.1428	1.01	< 0.001[a]
Fasting C-peptide (pmol/mL)	1.1976	0.0753	3.31	< 0.001[a]
Fasting insulin (μU/mL)[1]	0.9646	0.059	1.02	< 0.001[a]

[1]Log transformation was applied to this factor before including in regression model. [a]*P* < 0.05. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; FPG: Fasting plasma glucose; GGT: Gamma glutamyl transferase; HbA1c: Glycated hemoglobin; TIBC: Total iron-binding capacity. [a]*P* < 0.05.

Table 3 Risk factors of non-alcoholic fatty liver disease from step-wise logistic regression

	Beta	Standard error	Odds ratio	Change in pseudo R^2	*P* value
Demographic					
Race/ethnicity					
White (non-Hispanic)	0.2969	0.1162	1.35	0.18%	0.011
Mexican American	0.5495	0.1207	1.73	0.57%	0.020
Body measurement					
Waist circumference (cm)	0.0308	0.0035	1.03	2.13%	< 0.001
Biochemistry tests					
Ferritin (ng/mL)	0.0013	0.0004	1.00	0.15%	< 0.001
Triglyceride (mg/dL)	0.0008	0.0004	1.00	0.32%	< 0.001
Liver chemistry					
ALT (U/L)[1]	0.5658	0.0875	1.76	1.16%	< 0.001
Total protein (g/dL)	0.5319	0.1045	1.70	0.72%	< 0.001
Diabetes testing profile					
HbA1c (%)[1]	0.9266	0.2492	2.53	0.38%	< 0.001
Fasting C-peptide (pmol/mL)	0.6009	0.0877	1.82	1.33%	< 0.001

[1]Log transformation was applied to this factor before including in regression model. Note: Pseudo R^2 = 16.68%; constant (Y-intercept) = 0.0000153; change in Pseudo R^2 is an incremental increase in Pseudo R^2 resulting from adding variable to model last. ALT: Alanine aminotransferase; HbA1c: Glycated hemoglobin.

response protein, ferritin concentrations increase during inflammation and may not reflect the size of total body iron stores[44]. Moreover, ferritin was found be associated with histologic severity and advanced fibrosis in patients

with NAFLD[46-48].

Other factors significantly associated with NAFLD include race/ethnicity (non-Hispanic white and Mexican American), and triglyceride level. Race/ethnicity were often found associated with obesity-related diseases in United States based population[49,50] Triglyceride is an important biomarker of cardiovascular disease risk[51], another condition highly interrelated with NAFLD.

There are several limitations in this study. First, the diagnosis of NAFLD in this study is based on the hepatic ultrasound results although liver biopsy remains the gold standard for the diagnosis of NAFLD. Second, the statistical analysis used is logistic regression. Since the relationship among these factors are complex, inter-related, and non-linear, linearity assumptions embedded in logistic regression may not be able to address all aspects of NAFLD. Furthermore, given a pseudo R^2 of 16.68%, only 16.68% of variation can be explained by multivariate model in Table 3.

In conclusion, NAFLD is associated with many conditions and factors. Three most important factors from multivariate model in this study are waist circumference, fasting C-peptide, and ALT. Further study is needed to validate the clinical utility of C-peptide in diagnosis or monitoring insulin resistance in NAFLD patients.

ARTICLE HIGHLIGHTS

Research background
Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the United States. Additionally, NAFLD can progress to more severe liver diseases, such as non-alcoholic steatohepatitis, cirrhosis, and hepatocellular carcinoma. Many factors were found to be independently associated with NAFLD and/or severity of liver fibrosis in NAFLD. Nevertheless, there is limited evidence of the association between NAFLD and C-peptide.

Research motivation
Among many risk factors that are associated with NAFLD, obesity and insulin resistance are probably the most well-known ones. C-peptide levels can be used to measure insulin secretion and a surrogate marker of insulin resistance. However, C-peptide is not routinely used in clinical practice to diagnose type 2 diabetes or monitor insulin resistance status in NAFLD.

Research objectives
The objective of this study was to determine if fasting C-peptide is independently associated with NAFLD using multivariate analysis in the United States general population.

Research methods
Using the National Health and Nutrition Examination Survey 1988-1994, NAFLD participants aged 20 or greater without any other liver diseases were included in this study. The participants with excessive alcohol intake (> 2 drinks per day for males and > 1 drink per day for female) were excluded from the study. C-peptide and 27 other factors known to be associated with NAFLD (*e.g.*, age, gender, body mass index, waist circumference, race/ethnicity, liver chemistries, and other diabetes tests) were selected as predictors in regression model. Univariate logistic regression and multivariate step-wise logistic regression were used to determine if the significant predictors of NAFLD, respectively.

Research results
There were 3235 participants (*n* = 3235) that passed the exclusion criteria. Based on ultrasound findings, 817 (25.26%) participants were classified as

NAFLD. Twenty-four variables were significantly associated with NAFLD in univariate level; the *P*-value of these significant factors mostly below 0.001. Using multivariate analysis, we found 9 out of 24 factors to be significantly associated with NAFLD. Ranked by $\lrcorner R^2$, the top three factors ranked are waist circumference (OR = 1.03, $\lrcorner R^2$ = 2.13%, *P* < 0.001), C-peptide level (OR = 1.82, $\lrcorner R^2$ = 1.33%, *P* < 0.001), and log$_e$ of ALT (OR = 1.76, $\lrcorner R^2$ = 1.16%, *P* < 0.001). The pseudo R^2 of the multivariate model is 16.68%.

Research conclusions
C-peptide is the second most important predictor of NAFLD in United States population after waist circumference.

Research perspectives
Further prospective research is needed to validate the clinical utility of fasting C-peptide in diagnosis or monitoring insulin resistance in NAFLD patients. Moreover, C-peptide should be considered as a potential factor for calculative liver scores to evaluate the fibrosis level.

REFERENCES

1   **Fraser A**, Longnecker MP, Lawlor DA. Prevalence of elevated alanine aminotransferase among US adolescents and associated factors: NHANES 1999-2004. *Gastroenterology* 2007; **133**: 1814-1820 [PMID: 18054554 DOI: 10.1053/j.gastro.2007.08.077]

2   **Vernon G**, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Aliment Pharmacol Ther* 2011; **34**: 274-285 [PMID: 21623852 DOI: 10.1111/j.1365-2036.2011.04724.x]

3   **Williams CD**, Stengel J, Asike MI, Torres DM, Shaw J, Contreras M, Landt CL, Harrison SA. Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. *Gastroenterology* 2011; **140**: 124-131 [PMID: 20858492 DOI: 10.1053/j.gastro.2010.09.038]

4   **Beste LA**, Leipertz SL, Green PK, Dominitz JA, Ross D, Ioannou GN. Trends in burden of cirrhosis and hepatocellular carcinoma by underlying liver disease in US veterans, 2001-2013. *Gastroenterology* 2015; **149**: 1471-1482.e5; quiz e17-8 [PMID: 26255044 DOI: 10.1053/j.gastro.2015.07.056]

5   **Rinella ME**. Nonalcoholic fatty liver disease: a systematic review. *JAMA* 2015; **313**: 2263-2273 [PMID: 26057287 DOI: 10.1001/jama.2015.5370]

6   **Buzzetti E**, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Metabolism* 2016; **65**: 1038-1048 [PMID: 26823198 DOI: 10.1016/j.metabol.2015.12.012]

7   **Da Silva HE**, Teterina A, Comelli EM, Taibi A, Arendt BM, Fischer SE, Lou W, Allard JP. Nonalcoholic fatty liver disease is associated with dysbiosis independent of body mass index and insulin resistance. *Sci Rep* 2018; **8**: 1466 [PMID: 29362454 DOI: 10.1038/s41598-018-19753-9]

8   **Ruhl CE**, Everhart JE. Fatty liver indices in the multiethnic United States National Health and Nutrition Examination Survey. *Aliment Pharmacol Ther* 2015; **41**: 65-76 [PMID: 25376360 DOI: 10.1111/apt.13012]

9   **Angulo P**, Hui JM, Marchesini G, Bugianesi E, George J, Farrell GC, Enders F, Saksena S, Burt AD, Bida JP, Lindor K, Sanderson SO, Lenzi M, Adams LA, Kench J, Therneau TM, Day CP. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. *Hepatology* 2007; **45**: 846-854 [PMID: 17393509 DOI: 10.1002/hep.21496]

10   **Harrison SA**, Oliver D, Arnold HL, Gogia S, Neuschwander-Tetri BA. Development and validation of a simple NAFLD clinical scoring system for identifying patients without advanced disease. *Gut* 2008; **57**: 1441-1447 [PMID: 18390575 DOI: 10.1136/gut.2007.146019]

11   **Ma H**, Xu C, Xu L, Yu C, Miao M, Li Y. Independent association of HbA1c and nonalcoholic fatty liver disease in an elderly Chinese

population. *BMC Gastroenterol* 2013; **13**: 3 [PMID: 23294935 DOI: 10.1186/1471-230X-13-3]

12 **Van Cauter E**, Mestrez F, Sturis J, Polonsky KS. Estimation of insulin secretion rates from C-peptide levels. Comparison of individual and standard kinetic parameters for C-peptide clearance. *Diabetes* 1992; **41**: 368-377 [PMID: 1551497 DOI: 10.2337/diabetes.41.3.368]

13 **Chen M**, Li T, Zhang R, Li L, Lu J, Bao Y, Jia W. [Clinical features of non-alcoholic fatty liver disease and its relationship with serum C-peptide levels in patients with latent autoimmune diabetes in adults]. *Zhonghua Yi Xue Za Zhi* 2015; **95**: 3575-3578 [PMID: 26813367]

14 **Tricò D**, Caprio S, Umano GR, Pierpont B, Nouws J, Galderisi A, Kim G, Mata MM, Santoro N. Metabolic Features of Nonalcoholic Fatty Liver (NAFL) in Obese Adolescents: Findings from a Multi-ethnic Cohort. *Hepatology* 2018; [PMID: 29665034 DOI: 10.1002/hep.30035]

15 **Leighton E**, Sainsbury CA, Jones GC. A Practical Review of C-Peptide Testing in Diabetes. *Diabetes Ther* 2017; **8**: 475-487 [PMID: 28484968 DOI: 10.1007/s13300-017-0265-4]

16 **Wang L**, Lin P, Ma A, Zheng H, Wang K, Li W, Wang C, Zhao R, Liang K, Liu F, Hou X, Song J, Lu Y, Zhu P, Sun Y, Chen L. C-Peptide Is Independently Associated with an Increased Risk of Coronary Artery Disease in T2DM Subjects: A Cross-Sectional Study. *PLoS One* 2015; **10**: e0127112 [PMID: 26098780 DOI: 10.1371/journal.pone.0127112]

17 **Li Y**, Li Y, Meng L, Zheng L. Association between serum C-peptide as a risk factor for cardiovascular disease and high-density lipoprotein cholesterol levels in nondiabetic individuals. *PLoS One* 2015; **10**: e112281 [PMID: 25559358 DOI: 10.1371/journal.pone.0112281]

18 **Gonzalez-Mejia ME**, Porchia LM, Torres-Rasgado E, Ruiz-Vivanco G, Pulido-Pérez P, Báez-Duarte BG, Pérez-Fuentes R. C-Peptide Is a Sensitive Indicator for the Diagnosis of Metabolic Syndrome in Subjects from Central Mexico. *Metab Syndr Relat Disord* 2016; **14**: 210-216 [PMID: 26863426 DOI: 10.1089/met.2015.0067]

19 **National Center for Health Statistics**. Third National Health and Nutrition Examination Survey Data (NHANES III). Accessed May 29, 2018 Available from: URL: https://www.cdc.gov/nchs/nhanes/nhanes3.htm

20 **National Center for Health Statistics**. Third National Health and Nutrition Examination Survey: Hepatic Steatosis Ultrasound Images Assessment Procedures Manual. Accessed May 29, 2018 Available from: URL: https://www.cdc.gov/nchs/data/nhanes/nhanes3/hepatic_steatosis_ultrasound_procedures_manual.pdf

21 **Jensen MD**, Ryan DH, Apovian CM, Ard JD, Comuzzie AG, Donato KA, Hu FB, Hubbard VS, Jakicic JM, Kushner RF, Loria CM, Millen BE, Nonas CA, Pi-Sunyer FX, Stevens J, Stevens VJ, Wadden TA, Wolfe BM, Yanovski SZ; American College of Cardiology/American Heart Association Task Force on Practice Guidelines; Obesity Society. 2013 AHA/ACC/TOS guideline for the management of overweight and obesity in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines and The Obesity Society. *J Am Coll Cardiol* 2014; **63**: 2985-3023 [PMID: 24239920 DOI: 10.1016/j.jacc.2013.11.004]

22 **Dimitriadis K**, Tsioufis C, Mazaraki A, Liatakis I, Koutra E, Kordalis A, Kasiakogias A, Flessas D, Tentolouris N, Tousoulis D. Waist circumference compared with other obesity parameters as determinants of coronary artery disease in essential hypertension: a 6-year follow-up study. *Hypertens Res* 2016; **39**: 475-479 [PMID: 26865004 DOI: 10.1038/hr.2016.8]

23 **Grundy SM**, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith SC Jr, Spertus JA, Costa F; American Heart Association; National Heart, Lung, and Blood Institute. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation* 2005; **112**: 2735-2752 [PMID: 16157765 DOI: 10.1161/CIRCULATIONAHA.105.169404]

24 **Janssen I**, Katzmarzyk PT, Ross R. Waist circumference and not body mass index explains obesity-related health risk. *Am J Clin Nutr* 2004; **79**: 379-384 [PMID: 14985210 DOI: 10.1093/ajcn/79.3.379]

25 **Lindström J**, Tuomilehto J. The diabetes risk score: a practical tool to predict type 2 diabetes risk. *Diabetes Care* 2003; **26**: 725-731 [PMID: 12610029 DOI: 10.2337/diacare.26.3.725]

26 **Pazderska A**, Kyaw Tun T, Phelan N, McGowan A, Sherlock M, Behan L, Boran G, Gibney J. In women with PCOS, waist circumference is a better surrogate of glucose and lipid metabolism than disease status per se. *Clin Endocrinol* (Oxf) 2018; **88**: 565-574 [PMID: 29285778 DOI: 10.1111/cen.13542]

27 **Pouliot MC**, Després JP, Lemieux S, Moorjani S, Bouchard C, Tremblay A, Nadeau A, Lupien PJ. Waist circumference and abdominal sagittal diameter: best simple anthropometric indexes of abdominal visceral adipose tissue accumulation and related cardiovascular risk in men and women. *Am J Cardiol* 1994; **73**: 460-468 [PMID: 8141087 DOI: 10.1016/0002-9149(94)90676-9]

28 **Gaggini M**, Morelli M, Buzzigoli E, DeFronzo RA, Bugianesi E, Gastaldelli A. Non-alcoholic fatty liver disease (NAFLD) and its connection with insulin resistance, dyslipidemia, atherosclerosis and coronary heart disease. *Nutrients* 2013; **5**: 1544-1560 [PMID: 23666091 DOI: 10.3390/nu5051544]

29 **Asrih M**, Jornayvaz FR. Inflammation as a potential link between nonalcoholic fatty liver disease and insulin resistance. *J Endocrinol* 2013; **218**: R25-R36 [PMID: 23833274 DOI: 10.1530/JOE-13-0201]

30 **American Diabetes Association**. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2014; **37** Suppl 1: S81-S90 [PMID: 24357215 DOI: 10.2337/dc14-S081]

31 **Okabayashi T**, Shima Y, Sumiyoshi T, Kozuki A, Ito S, Ogawa Y, Kobayashi M, Hanazaki K. Diagnosis and management of insulinoma. *World J Gastroenterol* 2013; **19**: 829-837 [PMID: 23430217 DOI: 10.3748/wjg.v19.i6.829]

32 **Waickus CM**, de Bustros A, Shakil A. Recognizing factitious hypoglycemia in the family practice setting. *J Am Board Fam Pract* 1999; **12**: 133-136 [PMID: 10220236 DOI: 10.3122/jabfm.12.2.133]

33 **Hattersley A**, Bruining J, Shield J, Njolstad P, Donaghue K; International Society for Pediatric and Adolescent Diabetes. ISPAD Clinical Practice Consensus Guidelines 2006-2007. The diagnosis and management of monogenic diabetes in children. *Pediatr Diabetes* 2006; **7**: 352-360 [PMID: 17212604 DOI: 10.1111/j.1399-5448.2006.00217.x]

34 **Stenström G**, Gottsäter A, Bakhtadze E, Berger B, Sundkvist G. Latent autoimmune diabetes in adults: definition, prevalence, beta-cell function, and treatment. *Diabetes* 2005; **54** Suppl 2: S68-S72 [PMID: 16306343 DOI: 10.2337/diabetes.54.suppl_2.S68]

35 **Francque SM**, Verrijken A, Mertens I, Hubens G, Van Marck E, Pelckmans P, Michielsen P, Van Gaal L. Noninvasive assessment of nonalcoholic fatty liver disease in obese or overweight patients. *Clin Gastroenterol Hepatol* 2012; **10**: 1162-1168; quiz e87 [PMID: 22796457 DOI: 10.1016/j.cgh.2012.06.019]

36 **Hui JM**, Hodge A, Farrell GC, Kench JG, Kriketos A, George J. Beyond insulin resistance in NASH: TNF-alpha or adiponectin? *Hepatology* 2004; **40**: 46-54 [PMID: 15239085 DOI: 10.1002/hep.20280]

37 **Chitturi S**, Abeygunasekera S, Farrell GC, Holmes-Walker J, Hui JM, Fung C, Karim R, Lin R, Samarasinghe D, Liddle C, Weltman M, George J. NASH and insulin resistance: Insulin hypersecretion and specific association with the insulin resistance syndrome. *Hepatology* 2002; **35**: 373-379 [PMID: 11826411 DOI: 10.1053/jhep.2002.30692]

38 **Hua X**, Li M, Pan F, Xiao Y, Cui W, Hu Y. Non-alcoholic fatty liver disease is an influencing factor for the association of SHBG with metabolic syndrome in diabetes patients. *Sci Rep* 2017; **7**: 14532 [PMID: 29109457 DOI: 10.1038/s41598-017-15232-9]

39 **Kawachi I**, Robinson GM, Stace NH. A combination of raised serum AST:ALT ratio and erythrocyte mean cell volume level detects excessive alcohol consumption. *N Z Med J* 1990; **103**: 145-148 [PMID: 2342668]

40 **Kwo PY**, Cohen SM, Lim JK. ACG Clinical Guideline: Evaluation of Abnormal Liver Chemistries. *Am J Gastroenterol* 2017; **112**:

18-35 [PMID: 27995906 DOI: 10.1038/ajg.2016.517]

41 **Nyblom H**, Berggren U, Balldin J, Olsson R. High AST/ALT ratio may indicate advanced alcoholic liver disease rather than heavy drinking. *Alcohol Alcohol* 2004; **39**: 336-339 [PMID: 15208167 DOI: 10.1093/alcalc/agh074]

42 **Short MW**, Domagalski JE. Iron deficiency anemia: evaluation and management. *Am Fam Physician* 2013; **87**: 98-104 [PMID: 23317073]

43 **Kell DB**, Pretorius E. Serum ferritin is an important inflammatory disease marker, as it is mainly a leakage product from damaged cells. *Metallomics* 2014; **6**: 748-773 [PMID: 24549403 DOI: 10.1039/c3mt00347g]

44 **Abitbol V**, Borderie D, Polin V, Maksimovic F, Sarfati G, Esch A, Tabouret T, Dhooge M, Dreanic J, Perkins G, Coriat R, Chaussade S. Diagnosis of Iron Deficiency in Inflammatory Bowel Disease by Transferrin Receptor-Ferritin Index. *Medicine* (Baltimore) 2015; **94**: e1011 [PMID: 26131803 DOI: 10.1097/MD.0000000000001011]

45 **Mecklenburg I**, Reznik D, Fasler-Kan E, Drewe J, Beglinger C, Hruz P; Swiss IBD Cohort Study Group. Serum hepcidin concentrations correlate with ferritin in patients with inflammatory bowel disease. *J Crohns Colitis* 2014; **8**: 1392-1397 [PMID: 24825446 DOI: 10.1016/j.crohns.2014.04.008]

46 **Sabrina N**, Bai CH, Chang CC, Chien YW, Chen JR, Chang JS. Serum Iron:Ferritin Ratio Predicts Healthy Body Composition and Reduced Risk of Severe Fatty Liver in Young Adult Women. *Nutrients* 2017; **9** [PMID: 28777296 DOI: 10.3390/nu9080833]

47 **Parikh P**, Patel J, Ingle M, Sawant P. Serum ferritin levels predict histological severity in patients with nonalcoholic fatty liver disease in India. *Indian J Gastroenterol* 2015; **34**: 200-208 [PMID: 26108652 DOI: 10.1007/s12664-015-0572-5]

48 **Kowdley KV**, Belt P, Wilson LA, Yeh MM, Neuschwander-Tetri BA, Chalasani N, Sanyal AJ, Nelson JE; NASH Clinical Research Network. Serum ferritin is an independent predictor of histologic severity and advanced fibrosis in patients with nonalcoholic fatty liver disease. *Hepatology* 2012; **55**: 77-85 [PMID: 21953442 DOI: 10.1002/hep.24706]

49 **Schmiegelow MD**, Hedlin H, Mackey RH, Martin LW, Vitolins MZ, Stefanick ML, Perez MV, Allison M, Hlatky MA. Race and ethnicity, obesity, metabolic health, and risk of cardiovascular disease in postmenopausal women. *J Am Heart Assoc* 2015; **4** [PMID: 25994446 DOI: 10.1161/JAHA.114.001695]

50 **Davidson MB**, Schriger DL. Effect of age and race/ethnicity on HbA1c levels in people without known diabetes mellitus: implications for the diagnosis of diabetes. *Diabetes Res Clin Pract* 2010; **87**: 415-421 [PMID: 20061043 DOI: 10.1016/j.diabres.2009.12.013]

51 **Nordestgaard BG**, Varbo A. Triglycerides and cardiovascular disease. *Lancet* 2014; **384**: 626-635 [PMID: 25131982 DOI: 10.1016/S0140-6736(14)61177-6]

ORIGINAL ARTICLE

Clinical Trials Study

Vascular anatomy of inferior mesenteric artery in laparoscopic radical resection with the preservation of left colic artery for rectal cancer

Ke-Xin Wang, Zhi-Qiang Cheng, Zhi Liu, Xiao-Yang Wang, Dong-Song Bi

Ke-Xin Wang, Zhi-Qiang Cheng, Zhi Liu, Xiao-Yang Wang, Dong-Song Bi, Department of General Surgery, Qilu Hospital of Shandong University, Jinan 250012, Shandong Province, China

ORCID number: Ke-Xin Wang (0000-0002-9875-4059); Zhi-Qiang Cheng (0000-0002-6916-747X); Zhi Liu (0000-0002-1888-1550); Xiao-Yang Wang (0000-0001-5081-6321); Dong-Song Bi (0000-0002-6839-5067).

Author contributions: Wang KX and Bi DS designed the study and wrote the manuscript; Wang KX and Liu Z instructed the whole study and prepared the figures; Wang KX and Wang XY collected and analyzed the data; Wang KX, Cheng ZQ, Liu Z, and Bi DS performed the operations; all authors have approved the final version of the manuscript.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Qilu Hospital of Shandong University.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: All authors declare there are no competing interests in this study.

Data sharing statement: No additional data are available.

CONSORT 2010 statement: The experimental method of this study was not a randomized controlled trial.

Abstract

AIM
To investigate the vascular anatomy of inferior mesenteric artery (IMA) in laparoscopic radical resection with the preservation of left colic artery (LCA) for rectal cancer.

METHODS
A total of 110 patients with rectal cancer who underwent laparoscopic surgical resection with preservation of the LCA were retrospectively reviewed. A 3D vascular reconstruction was performed before each surgical procedure to assess the branches of the IMA. During surgery, the relationship among the IMA, LCA, sigmoid artery (SA) and

superior rectal artery (SRA) was evaluated, and the length from the origin of the IMA to the point of branching into the LCA or common trunk of LCA and SA was measured. The relationship between inferior mesenteric vein (IMV) and LCA was also evaluated.

RESULTS
Three vascular types were identified in this study. In type A, LCA arose independently from IMA (46.4%, *n* = 51); in type B, LCA and SA branched from a common trunk of the IMA (23.6%, *n* = 26); and in type C, LCA, SA, and SRA branched at the same location (30.0%, *n* = 33). The difference in the length from the origin of IMA to LCA was not statistically significant among the three types. LCA was located under the IMV in 61 cases and above the IMV in 49 cases.

CONCLUSION
The vascular anatomy of the IMA and IMV is essential for laparoscopic radical resection with preservation of the LCA for rectal cancer. To recognize different branches of the IMA is necessary for the resection of lymph nodes and dissection of vessels.

Key words: Inferior mesenteric artery; Left colic artery; Rectal cancer; Laparoscopic

Core tip: One hundred and ten patients who underwent laparoscopic surgical resection with preservation of left colic artery (LCA) for rectal cancer were retrospectively reviewed. The 3D reconstruction of the vasculature was performed before surgical procedures. The types of branch vessels of inferior mesenteric artery (IMA) were classified. Furthermore, in the operations, relationships between the IMA, sigmoid artery (SA), LCA and superior rectal artery (SRA) were evaluated. The relationship between LCA and inferior mesenteric vein (IMV) was also evaluated. To recognize different branches of the IMA is necessary for the resection of lymph nodes and dissection of vessels during laparoscopic radical resection of rectal cancer.

INTRODUCTION

In laparoscopic radical resection for rectal cancer, high-tie of the inferior mesenteric artery (IMA) at its origin is essential for *en bloc* lymph node dissection. Some researchers have demonstrated the clinical significance of lymph node dissection from the origin of the IMA for postoperative staging and prognosis[1]. However, high tie at the origin of the IMA may lead to postoperative poor anastomotic perfusion, which increases the incidence of anastomotic leakage[2,3]. Many surgeons prefer a low-tie technique, which ligates the IMA while preserving the left colic artery (LCA) after lymph nodes around the IMA were dissected[4,5]. A clear understanding of the vascular anatomy of the IMA and inferior mesenteric vein (IMV) is essential for this low-tie procedure. However, there are multiple types of branch vessels originating from the IMA that makes this surgical procedure technically demanding[6]. We studied the vascular anatomy of the IMA to safely and effectively dissect the lymph nodes around the IMA while preserving the LCA in a laparoscopic procedure for rectal cancer.

MATERIALS AND METHODS

Patient data

Following approval of the Ethics Committee on Scientific Research of our hospital, the records of 110 patients, who underwent laparoscopic surgical resection with preservation of the LCA for rectal cancer from March 2016 to November 2017 were retrospectively analyzed. The research participants were recruited from the Department of General Surgery of Qilu Hospital, a teaching hospital of Shandong University in Shandong, China. The data of patient age, sex ratio, BMI, and histopathological stage were observed and compared.

Preoperative assessment of vascular anatomy by 3D reconstruction

The 3D reconstruction of the vascular anatomy was performed in all cases before surgery. The types of branch vessels of the IMA as well as the length from the origin of the IMA to the LCA were determined.

Surgical procedure

Laparoscopic surgery was performed by a single operating team consisting of two senior surgeons and three staff surgeons. During the operation, the patients were placed in lithotomy position. Multi-incision laparoscopic surgery was performed. That was, we placed five ports including the optical port. There were the first 10 mm trocar in the umbilicus as the optical port, another 12-mm trocar, and three 5-mm trocars. We established pneumoperitoneum and maintained abdominal pneumoperitoneum pressure at 12 mmHg. Sharp dissections were performed using a laparoscopic ultrasound knife. First, the small intestine was pulled on its cephalic side to allow for sufficient surgical space. Second, the sigmoid colon mesentery was mobilized with medial to lateral approach up to the origin of the IMA. Lymphous and adipic tissues were resected along the IMA down to the point of branching into the LCA or common trunk of LCA and sigmoid artery (SA). The dissection was then conducted from the LCA until the

Table 1 Patient characteristic data

Factors	Type A (*n* = 51)	Type B (*n* = 26)	Type C (*n* = 33)	Statistical value	*P* value
Age (yr, mean ± SD)	58.6 ± 14.1	60.4 ± 15.0	62.3 ± 12.9	F = 2.881	0.06
Male, *n* (%)	31 (60.8)	15 (57.7)	18 (54.5)	Pearson χ^2 = 0.324	0.85
BMI (kg/m²)	25.3 ± 5.4	24.8 ± 4.9	25.1 ± 4.6	F = 0.331	0.72
Stage (UICC 8th)				Fisher = 1.510	0.84
I	7	5	6		
II	26	10	16		
III	18	11	11		

P < 0.05. BMI: Body mass index; UICC: Union for International Cancer Control.
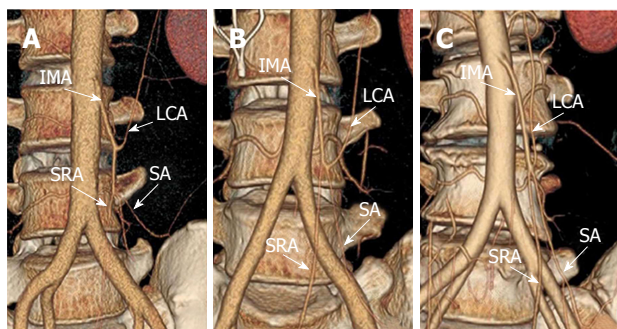

Figure 1 Preoperative 3D reconstruction of inferior mesenteric artery, left colic artery, sigmoid artery and superior rectal artery. A: Type A, LCA arose independently from IMA. B: type B, LCA and SA branched from a common trunk from IMA. C: type C, LCA, SA, and SRA branched off at the same point. IMA: Inferior mesenteric artery; LCA: Left colic artery; SA: Sigmoid artery; SRA: Superior rectal artery.

Figure 2 Inferior mesenteric artery, left colic artery, sigmoid artery and superior rectal artery in laparoscopic operation. A: Type A, LCA arose independently from IMA. B: type B, LCA and SA branched from a common trunk from IMA. C: type C, LCA, SA, and SRA branched off at the same point. IMA: Inferior mesenteric artery; LCA: Left colic artery; SA: Sigmoid artery; SRA: Superior rectal artery.
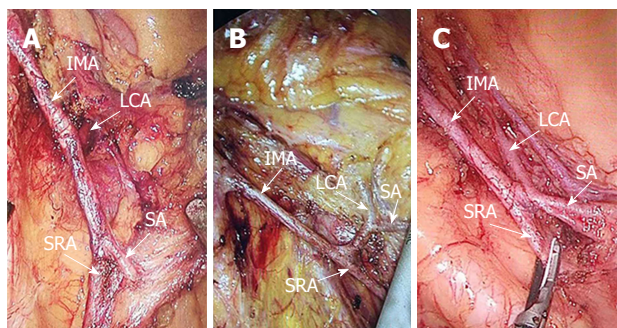
lymph nodes and the incidence of metastasis to station 253 nodes were recorded. Furthermore, the incidence of anastomotic bleeding and anastomotic leakage, and the length of postoperative hospital stay were investigated.

Statistical analysis
All experimental data were analyzed using the SPSS software version 17.0 (SPSS Inc., Chicago, IL, United States). There were three groups of samples in this study. The three groups were compared by single factor analysis of variance (single factor ANOVA) and homogeneity of variance. The comparison between the two groups was used on the LSD method. The count data were represented by the value/percentage (%), and the three groups were compared with the chi square test and Fisher accurate test. When the theoretical number *T* < 5, the Fisher test value was used, if not, the Pearson chi square test was used.

RESULTS

Clinical characteristics of the patients
The data of patient age, sex ratio, BMI, and histopathological stage are shown in Table 1. There were no statistically significant differences among the three types.

Vascular study
IMA, LCA, SA, and SRA were studied in 110 cases by preoperative 3D reconstruction of the vascular anatomy (Figure 1A-C) and laparoscopic surgery (Figure 2A-C). Three vascular types were identified in the study: type A (Figures 1A, 2A, and 3A), LCA arose independently from the IMA (46.4%, *n* = 51); type B (Figures 1B, 2B, and 3B), LCA and SA branched from a common trunk of the IMA (23.6%, *n* = 26); and type C (Figures 1C, 2C, and 3C), LCA, SA, and SRA branched at the same location (30.0%, *n* = 33). The length from the origin of the IMA to the LCA is displayed in Table 2. There was no statistically significant difference among the three types.

In laparoscopic surgery, the relationship between the location of LCA and IMV was observed. The LCA was located under the IMV in 61 cases and above the IMV in 49 cases. The ratio regarding the location of the LCA under the IMV in the three types was similar (Table 2).

Surgical outcome
As shown in Table 3, the data of operating time, blood

IMV could be identified. The IMV was exposed to the plane of the origin of the IMA. After the dissection, the vessels were ligated and cut with the preservation of the LCA. The superior hypogastric nerve and left ureter were carefully preserved during the procedure.

During surgery, the relationship among the IMA, LCA, SA, and superior rectal artery (SRA) was evaluated, and the length from the origin of the IMA to the point of branching into the LCA or common trunk of LCA and SA was measured. In addition, the relationship between LCA and IMV was also evaluated. The number of resected

Table 2 Results of vascular anatomy

Factors	Type A (*n* = 51)	Type B (*n* = 26)	Type C (*n* = 33)	Statistical value	*P* value
Length from the IMA to the LCA (mm) (mean ± SD)	35.2 ± 8.7	37.8 ± 9.4	41.3 ± 11.5	*F* = 1.976	0.144
No. of LCA under IMV	27 (52.9%)	14 (53.8%)	20 (60.6%)	Pearson χ^2 = 0.512	0.802

P < 0.05. IMA: Inferior mesenteric artery; IMV: Inferior mesenteric vein; LCA: Left colic artery.

Table 3 Surgical outcome of patients

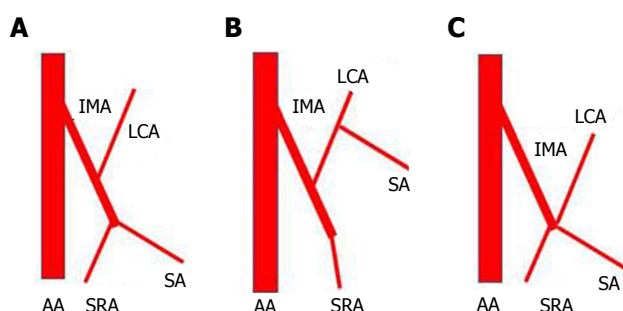|	Type A (*n* = 51)	Type B (*n* = 26)	Type C (*n* = 33)	Statistical value	*P* value
Operation time (min)	153.4 ± 26.8	168.7 ± 31.6	161.4 ± 25.8	*F* = 1.618	0.20
Blood loss (g)	37.5 ± 18.4	42.1 ± 17.7	39.6 ± 20.1	*F* = 1.383	0.26
Lymph node numbers (*n*, mean ± SD)	15.7 ± 8.3	17.2 ± 8.1	16.8 ± 9.0	*F* = 0.620	0.54
Metastasis to station 253 lymph nodes (*n*)	2	1	1	Fisher = 0.368	1.00
Anastomotic bleeding	1		1	Fisher = 0.930	0.10
Anastomotic leakage	1	1		Fisher = 1.407	0.72
Postoperative hospitalized days	9 (7-21)	9 (6-18)	9 (7-13)	χ^2 = 0.863	0.65

P < 0.05.

Figure 3 Vascular schematic diagram of inferior mesenteric artery, left colic artery, sigmoid artery and superior rectal artery. A: Type A, LCA arose independently from IMA. B: type B, LCA and SA branched from a common trunk from IMA. C: type C, LCA, SA, and SRA branched off at the same point. AA: Abdominal aorta; IMA: Inferior mesenteric artery; LCA: Left colic artery; SA: Sigmoid artery; SRA: Superior rectal artery.

loss, and the length of postoperative hospital stay were not statistically significant among the three groups. Additionally, there were no statistically significant differences in the dissected lymph node numbers. The incidence of metastasis to station 253 nodes was 4.5% (5 of 110). The postoperative complications included anastomotic bleeding in two cases and anastomotic leakage in two cases. No statistically significant difference was observed among the three groups.

DISCUSSION

In laparoscopic radical resection for rectal cancer, according to the location of the tie of the IMA, it is divided into the high-tie of the IMA at its origin and the low-tie of the IMA below the branch into the LCA with preservation of the LCA. Currently, there is still controversy regarding the indications for high-tie or low-tie approaches[7-9]. In traditional rectal cancer surgery, a high tie of the IMA is preferred. However, some anatomical studies suggest that anastomotic perfusion is diminished after the high-tie of the IMA[3]. Consequently, postoperative poor anastomotic perfusion increased the incidence of anastomotic leakage[10,11]. LCA can increase the blood supply of anastomotic and proximal colorectum. The Riolan artery arch is an anastomotic branch between ascending branches of the LCA and the left branches of the middle colonic artery. It is vital to the blood supply of the anastomotic and proximal colorectum. The Riolan arch exists in about 7.6% of the Chinese population[12]. In patients with absence of the Riolan arch, left hemicolon relies on blood supply from the IMA, and the high-tie technique causes ischemic changes of anastomotic stoma easily. Some Chinese surgeons have found that the absence of the Riolan arch is an independent risk factor for anastomotic leakage after laparoscopic radical resection of rectal cancer[13]. So the low-tie technique with preservation of the LCA to maintain the blood supply is recommended[14,15]. Furthermore, more and more research has indicated that the preservation of the LCA decreases the rate of anastomotic leakage[2,14,15].

A clear understanding of the vascular anatomy of the IMA and IMV is the essential knowledge required to conduct this surgical procedure. There are multiple types of branch vessels of the IMA that makes this surgery technically demanding. In our study, the branching patterns of the IMA can be divided into three groups by preoperative 3D vascular reconstruction and laparoscopic surgery (Figures 1 and 2). However, in other studies, there is a fourth pattern with the absence of the LCA[6,16]. Similarly, the relationship between the LCA and IMV was also observed. In 61 of the 110 cases, the LCA ran under the IMV. In these cases, during dissection of the LCA, extreme caution should be taken, and ligation of the SMV was performed first in order to avoid any damage to the IMV. Although there is still uncertainty with regard to the technical difficulty of the procedure, Sekimoto *et al*[17] have demonstrated that compared to the high-tie technique, the low-tie procedure did not prolong the

operating time and did not increase the amount of intra-operative bleeding. In the present study, compared with other previous studies on high ligation, the operating time was not prolonged significantly[17,18]. In addition, the incidence of postoperative complications was relatively low owing to the familiarity with anatomy and meticulous operation. According to our results, low-tie of the IMA with preservation of the LCA was safe and feasible.

Lymph node metastasis plays a crucial role in the prognosis of rectal cancer[18]. It was demonstrated that lymph node dissection around the IMA prolonged the survival of patients with lymph node metastasis. Low-tie of the IMA with preservation of the LCA was suspected to hinder the lymph nodes resection surrounding the IMA. A prospective study showed that the low-tie group had a similar number of lymph nodes dissected compared to the high-tie group[19]. Other studies compared the prognosis between high-tie and low-tie, and found that the overall survival (OS) and recurrence free survival (RFS) were similar between the two treatments, even in those with lymph node metastases[5]. In the present study, the number of lymph nodes dissected with the low-tie technique for rectal cancer (Table 1) was similar to other studies[1,6]. Nonetheless, our study did not include a high-tie control group, which is a major limitation to the results and prevents an effective discussion to be established to address these problems.

In conclusion, knowledge of the anatomy of the branch vessels originating from the IMA and the relationship between the IMA and IMV are essential in order to conduct a laparoscopic radical resection with preservation of the LCA for rectal cancer. To recognize the different branches of the IMA is necessary for the resection of lymph nodes and dissection of vessels.

ARTICLE HIGHLIGHTS

Research background
In laparoscopic radical resection for rectal cancer, according to the location of the tie of the inferior mesenteric artery (IMA), it is divided into the high-tie of the IMA at its origin and the low-tie of the IMA below the branch into the left colic artery (LCA) with preservation of the LCA. Currently, there is still controversy regarding the indications for high-tie or low-tie approaches.

Research motivation
In laparoscopic radical resection for rectal cancer, high-tie of the IMA at its origin is essential for *en bloc* lymph node dissection. We studied the vascular anatomy of the IMA to safely and effectively dissect the lymph nodes around the IMA while preserving the LCA in a laparoscopic procedure for rectal cancer.

Research objectives
We aimed to investigate the vascular anatomy of IMA in laparoscopic radical resection with the preservation of LCA for rectal cancer.

Research methods
The records of 110 patients, who underwent laparoscopic surgical resection with preservation of the LCA for rectal cancer from March 2016 to November 2017 were retrospectively analyzed. The research participants were recruited from the Department of General Surgery of Qilu Hospital, a teaching hospital of Shandong University in Shandong, China. A 3D vascular reconstruction was performed before each surgical procedure to assess the branches of the IMA.

During surgery, the relationship among the IMA, LCA, sigmoid artery (SA) and superior rectal artery (SRA) was evaluated.

Research results
IMA, LCA, SA, and SRA were studied in 110 cases by preoperative 3D reconstruction of the vascular anatomy and laparoscopic surgery. Three vascular types were identified in the study: type A, LCA arose independently from the IMA (46.4%, *n* = 51); type B, LCA and SA branched from a common trunk of the IMA (23.6%, *n* = 26); and type C, LCA, SA, and SRA branched at the same location (30.0%, *n* = 33). There was no statistically significant difference in the length from the origin of the IMA to the LCA among the three types. In laparoscopic surgery, the LCA was located under the IMV in 61 cases and above the IMV in 49 cases. The ratio regarding the location of the LCA under the IMV in the three types was similar. The data of operating time, blood loss, and the length of postoperative hospital stay were not statistically significant among the three types. Additionally, there were no statistically significant differences in the dissected lymph node numbers. The incidence of metastasis to station 253 nodes was 4.5% (5 of 110). The postoperative complications included anastomotic bleeding in two cases and anastomotic leakage in two cases.

Research conclusions
Knowledge of the anatomy of the branch vessels originating from the IMA and the relationship between the IMA and IMV are essential in order to conduct a laparoscopic radical resection with preservation of the LCA for rectal cancer. To recognize the different branches of the IMA is necessary for the resection of lymph nodes and dissection of vessels.

Research perspectives
We studied the vascular anatomy of the IMA to safely and effectively dissect the lymph nodes around the IMA while preserving the LCA in a laparoscopic procedure for rectal cancer. However, in the light of the limited evidence, the clinical benefit needs more high-quality RCT studies.

REFERENCES

1   **You X**, Wang Y, Chen Z, Li W, Xu N, Liu G, Zhao X, Huang C. [Clinical study of preserving left colic artery during laparoscopic total mesorectal excision for the treatment of rectal cancer]. *Zhonghua WeiChang WaiKe ZaZhi* 2017; **20**: 1162-1167 [PMID: 29130232]

2   **Hinoi T**, Okajima M, Shimomura M, Egi H, Ohdan H, Konishi F, Sugihara K, Watanabe M. Effect of left colonic artery preservation on anastomotic leakage in laparoscopic anterior resection for middle and low rectal cancer. *World J Surg* 2013; **37**: 2935-2943 [PMID: 24005279 DOI: 10.1007/s00268-013-2194-3]

3   **Komen N**, Slieker J, de Kort P, de Wilt JH, van der Harst E, Coene PP, Gosselink MP, Tetteroo G, de Graaf E, van Beek T, den Toom R, van Bockel W, Verhoef C, Lange JF. High tie versus low tie in rectal surgery: comparison of anastomotic perfusion. *Int J Colorectal Dis* 2011; **26**: 1075-1078 [PMID: 21445553 DOI: 10.1007/s00384-011-1188-6]

4   **Shen J**, Li M, Du Y, Xie D, Qu H, Zhang Y. Long-term outcomes of laparoscopic low anterior resection of rectal carcinoma with preservation of the left colic artery. *Zhonghua WeiChang WaiKe ZaZhi* 2017; **20**: 660-664 [PMID: 28643311]

5   **Yasuda K**, Kawai K, Ishihara S, Murono K, Otani K, Nishikawa T, Tanaka T, Kiyomatsu T, Hata K, Nozawa H, Yamaguchi H, Aoki S, Mishima H, Maruyama T, Sako A, Watanabe T. Level of arterial ligation in sigmoid colon and rectal cancer surgery. *World J Surg Oncol* 2016; **14**: 99 [PMID: 27036117 DOI: 10.1186/s12957-016-0819-3]

6   **Miyamoto R**, Nagai K, Kemmochi A, Inagawa S, Yamamoto M. Three-dimensional reconstruction of the vascular arrangement including the inferior mesenteric artery and left colic artery in laparoscope-assisted colorectal surgery. *Surg Endosc* 2016; **30**: 4400-4404 [PMID: 26850027 DOI: 10.1007/s00464-016-4758-4]

7   **Cirocchi R**, Trastulli S, Farinella E, Desiderio J, Vettoretto N, Parisi A, Boselli C, Noya G. High tie versus low tie of the inferior

mesenteric artery in colorectal cancer: a RCT is needed. *Surg Oncol* 2012; **21**: e111-e123 [PMID: 22770982 DOI: 10.1016/j.suronc.2012.04.004]

8   **Hida J**, Okuno K. High ligation of the inferior mesenteric artery in rectal cancer surgery. *Surg Today* 2013; **43**: 8-19 [PMID: 23052748 DOI: 10.1007/s00595-012-0359-6]

9   **Mari G**, Maggioni D, Costanzi A, Miranda A, Rigamonti L, Crippa J, Magistro C, Di Lernia S, Forgione A, Carnevali P, Nichelatti M, Carzaniga P, Valenti F, Rovagnati M, Berselli M, Cocozza E, Livraghi L, Origi M, Scandroglio I, Roscio F, De Luca A, Ferrari G, Pugliese R. "High or low Inferior Mesenteric Artery ligation in Laparoscopic low Anterior Resection: study protocol for a randomized controlled trial" (HIGHLOW trial). *Trials* 2015; **16**: 21 [PMID: 25623323 DOI: 10.1186/s13063-014-0537-5]

10   **Dworkin MJ**, Allen-Mersh TG. Effect of inferior mesenteric artery ligation on blood flow in the marginal artery-dependent sigmoid colon. *J Am Coll Surg* 1996; **183**: 357-360 [PMID: 8843265]

11   **Lim DR**, Hur H, Min BS, Baik SH, Kim NK. Colon Stricture After Ischemia Following a Robot-Assisted Ultra-Low Anterior Resection With Coloanal Anastomosis. *Ann Coloproctol* 2015; **31**: 157-162 [PMID: 26361618 DOI: 10.3393/ac.2015.31.4.157]

12   **Cheng BC**, Chang S, Huang J, Mao ZF, Wang ZW, Lu SQ, Wang TS, Wu XJ, Hu H, Xia J, Kang GJ, Xiao YG, Lin HQ. [Surgical anatomy of the colic vessels in Chinese and its influence on the operation of esophageal replacement with colon]. *Zhonghua Yi Xue Za Zhi* 2006; **86**: 1453-1456 [PMID: 16842695]

13   **Huang J**, Zhou J, Wan Y, Lin Y, Deng Y, Zhou Z, Qiu J, Wang J, Huang M. Influences of inferior mesenteric artery types and Riolan artery arcade absence on the incidence of anastomotic leakage after laparoscopic resection of rectal cancer. *Zhonghua WeiChang WaiKe ZaZhi* 2016; **19**: 1113-1118 [PMID: 27781246 DOI: 10.3760/cma.j.issn.1671-0274.2016.10.008]

14   **Napolitano AM**, Napolitano L, Costantini R, Ucchino S, Innocenti P. [Skeletization of the inferior mesenteric artery in colorectal surgery. Current considerations]. *G Chir* 1996; **17**: 185-189 [PMID: 8754557]

15   **Messinetti S**, Giacomelli L, Manno A, Finizio R, Fabrizio G, Granai AV, Busicchio P, Lauria V. Preservation and peeling of the inferior mesenteric artery in the anterior resection for complicated diverticular disease. *Ann Ital Chir* 1998; **69**: 479-482; discussion 482-483 [PMID: 9835123]

16   **Kobayashi M**, Morishita S, Okabayashi T, Miyatake K, Okamoto K, Namikawa T, Ogawa Y, Araki K. Preoperative assessment of vascular anatomy of inferior mesenteric artery by volume-rendered 3D-CT for laparoscopic lymph node dissection with left colic artery preservation in lower sigmoid and rectal cancer. *World J Gastroenterol* 2006; **12**: 553-555 [PMID: 16489667 DOI: 10.3748/wjg.v12.i4.553]

17   **Patroni A**, Bonnet S, Bourillon C, Bruzzi M, Zinzindohoué F, Chevallier JM, Douard R, Berger A. Technical difficulties of left colic artery preservation during left colectomy for colon cancer. *Surg Radiol Anat* 2016; **38**: 477-484 [PMID: 26526820 DOI: 10.1007/s00276-015-1583-8]

18   **Kanemitsu Y**, Hirai T, Komori K, Kato T. Survival benefit of high ligation of the inferior mesenteric artery in sigmoid colon or rectal cancer surgery. *Br J Surg* 2006; **93**: 609-615 [PMID: 16607682 DOI: 10.1002/bjs.5327]

19   **Sekimoto M**, Takemasa I, Mizushima T, Ikeda M, Yamamoto H, Doki Y, Mori M. Laparoscopic lymph node dissection around the inferior mesenteric artery with preservation of the left colic artery. *Surg Endosc* 2011; **25**: 861-866 [PMID: 20725744 DOI: 10.1007/s00464-010-1284-7]

Baishideng®

3 2>

9 771007 932045