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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.

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REVIEW

# Role of microRNAs in the main molecular pathways of hepatocellular carcinoma

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## 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.

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## 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, cardiovascular 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 demonstrated 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-

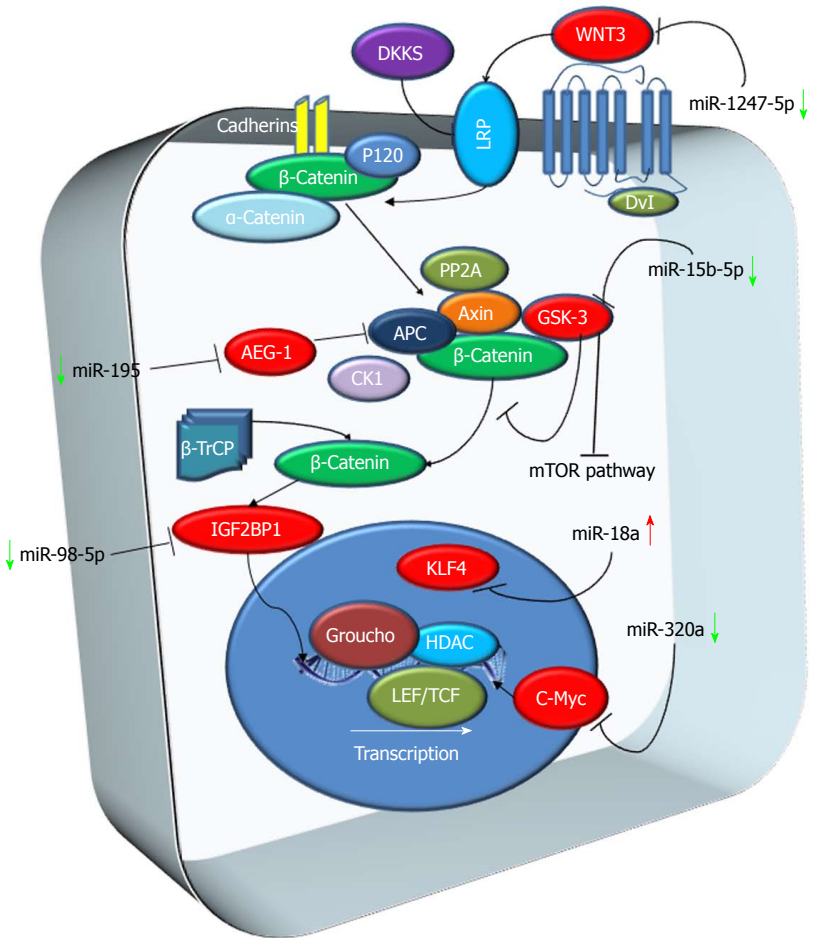


**Figure 1 Wnt signaling.** MicroRNAs deregulated in hepatocellular carcinoma and involved in Wnt signaling pathway.

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

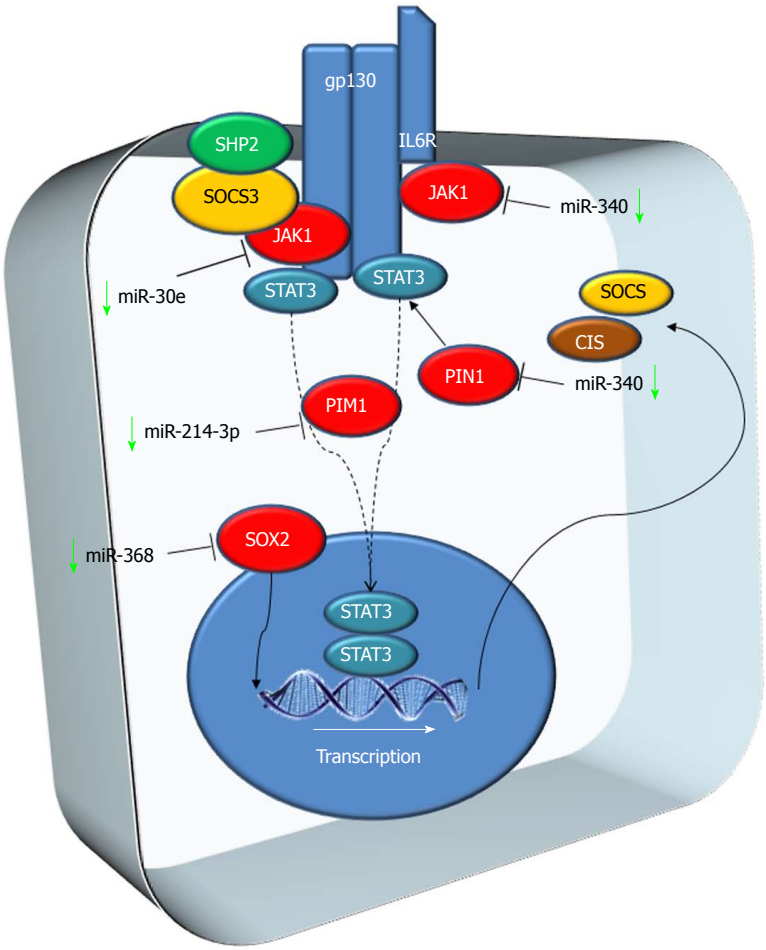


**Figure 2 JAK/STAT pathway.** MicroRNAs deregulated in hepatocellular carcinoma and involved in JSK/STAT pathway.

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

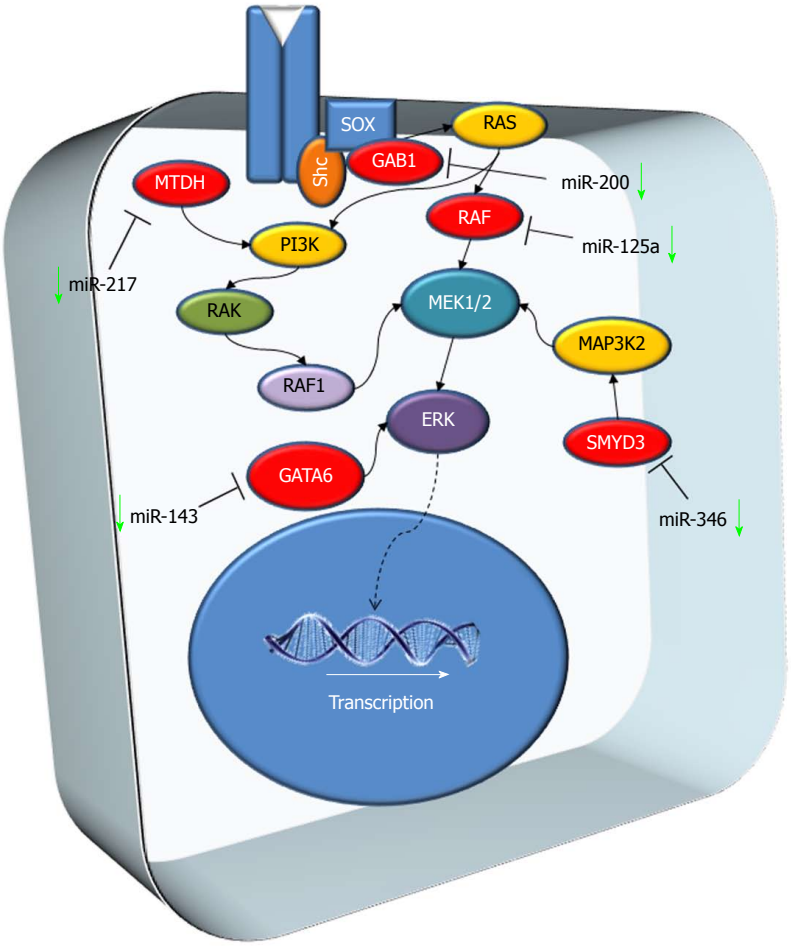


**Figure 3 MAPK cascade.** MicroRNAs deregulated in hepatocellular carcinoma and involved in MAPK cascade.

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.

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REVIEW

# Apoptosis and non-alcoholic fatty liver diseases

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## 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.

**Key words:** Apoptosis; Autophagy; c-Jun N-terminal kinase; Nonalcoholic fatty liver diseases; Nonalcoholic steatohepatitis



**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.

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, Moriyama M. Apoptosis and non-alcoholic fatty liver diseases. *World J Gastroenterol* 2018; 24(25): 2661-2672 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i25/2661.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i25.2661

## 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-3$\alpha$ and GSK-3$\beta$, 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 $\alpha$, 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] |

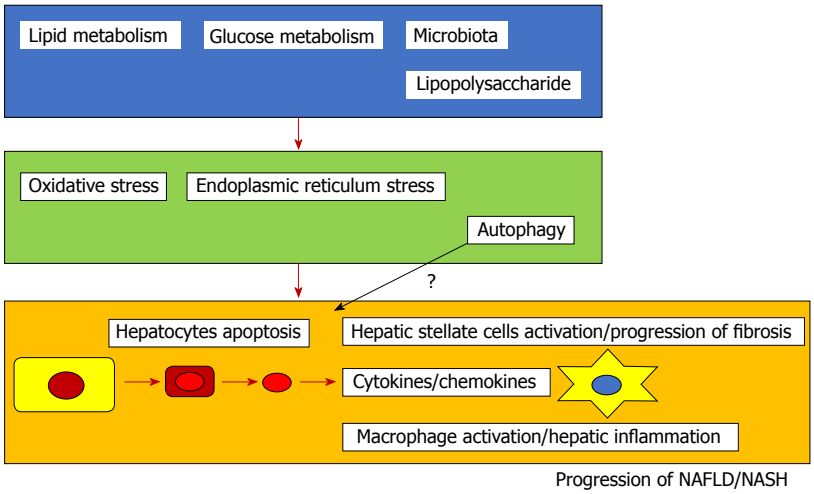


**Figure 1 Disease progression of nonalcoholic fatty liver diseases and nonalcoholic steatohepatitis.** The exact role of autophagy in nonalcoholic fatty liver diseases/nonalcoholic steatohepatitis remains unclear. NAFLD: Nonalcoholic fatty liver diseases; NASH: Nonalcoholic steatohepatitis.

## 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.

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REVIEW

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

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## 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.

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## 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<br>Inhibits the generation and activity of IL-1β, IL-4, IL-5, IL-8, granulocyte-macrophage colony stimulating factor, and TNF-α | [23] |
| Corticosteroids | Corticosteroids | | [24] |
| Immunosuppressants | Azathioprine<br>6-mercaptopurine<br>Cyclosporine A<br>Tacrolimus<br>Methotrexaten | Clinical remission<br>Mucosal healing | [25] |
| Antibiotics | Metronidazole<br>Ciprofloxacin | Decrease disease activity index<br>Maintain remission | [26] |
| Biological therapy | Infliximab<br>Adalimumab<br>Certolizumab | Neutralizes TNF-α<br>Reduces inflammation | [27] |

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 ($CD11b^+Gr\text{-}1^+$) 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 favorable 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 protective 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<br>Reduced TNF-α, IFN-γ, IL-1β and MPO activity | [34] |
| | *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<br>Increased IL-10; Decreased IL-1β<br>Engaged TLR2; Contained NOD2<br>Improved epithelial barrier | [31] |
| 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<br>Lowered MPO, NO, and MDA<br>Inhibited the expressions of TNF-α, IL-1β, IL-6 and iNOS | [46] |
| | Glucan from mushroom (*Pleurotus pulmonarius*) | Reduced histological damage | [47] |
| | Bacterial β-(1,3)-glucan | Reduced the expression of IL-1β<br>Reversed Treg reduction | [48] |
| Nanofiber | Cellulose nanofiber from seaweed | Decreased NK cell defects and IgA production | [49] |
| Prebiotics | Cellulose nanofiber from pear | Improved intestinal tissue injury<br>Suppressed the activation of NF-κB | [50] |
| | Fructooligosaccharides | Suppressed colon atrophy<br>Suppressed the activation of NF-κB | [51] |
| | Goat milk oligosaccharide | Decreased IFN-γ, IL-17, and TNF-α levels | [52] |
| | Inulin | Increased LAB population<br>Decreased inflammation<br>Improved mucosal damage<br>Decreased TNFα, COX-2, IL-2, and IL-6 | [53] |
| Vitamins | 1alpha,25-dihydroxyvitamin D3 | Suppressed TNF-α<br>Enhanced IL-10 production | [57] |
| | 1,25-dihydroxyvitamin D3 | Reduced IFN-γ | [58] |
| | Vitamin D3 | Increased CD+ T cells and IL-6<br>Protected mitochondria | [59] |
| | 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-$\alpha$, IFN-$\gamma$, IL-4, IL-1$\beta$ 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-$\alpha$ 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-$\alpha$, 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-$\kappa$B 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-$\alpha$, MAPK phosphorylation, and NF-$\kappa$B 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-$\kappa$B 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-$\kappa$B and IL-1$\beta$ 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-$\gamma$, 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<br>Reduced STAT1 and STAT6 | [68] |
| | *Cordiceps militaris* extract | Decreased epithelial damage<br>Suppressed iNOS and TNF-α mRNA expression | [70] |
| | *Inonotus obliquus* extract | Suppressed TNF-α, COX-2, and IFN-γ | [73] |
| | *Ganoderma lucidum* extract | Inhibited MAPK phosphorylation and NF-κB activation<br>Decreased histological score | [74] |
| 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<br>Blueberry extract | Modulated NF-κB and IL-1β signaling<br>Attenuated colon shortening | [79] |
| | | Suppressed pro-inflammatory cytokines | [80] |
| *Averrhoa bilimbi* | *Averrhoa bilimbi* L. extract | Prevented oxidation<br>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<br>Decreased TBARS concentration<br>Suppressed NF-κB and IL-1β | [84] |
| | Green algae extract | Suppressed colonic tissue damage<br>Downregulated IFN-γ and IL-4<br>Ameliorated colonic tissue damage<br>Decreased pro-inflammatory cytokines | |
| Phytochemicals | Apple polyphenols | Reduced COX-2 and TNF-α<br>Recovered transglutaminase protein | [85] |
| | Resveratrol | Suppressed NF-κB and TNF-α<br>Reduced clinical score | [86] |
| | Cardamonin | Reduced histopathological damage<br>Reduced iNOS, NF-κB, TNF-α, COX-2, and caspase-3<br>Suppressed IL-1β and TNF-α | [18] |
| | Ginsenoside Rg1 | Reduced colonic damage and DAI<br>Improved colon shortening and DAI | [90] |
| | Sulforaphane | Suppressed STAT3 expression | [91] |
| | Curcumin | Reduced TNF-α, IL-1β, and MPO<br>Attenuated morphological damage | [92] |

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.

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MINIREVIEWS

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

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## 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.

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## 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<br>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<br>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<br>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<br>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<br>N3 + I1: Nivolumab 3 mg/kg and ipilimumab 1 mg/kg every 3 wk<br>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%<br>N1+I3: ORR 24%, PD-L1≥ 1% ORR 40%, PD-L1< 1% ORR 22%<br>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)<br>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/m$^2$ day 1), and 5-FU (800 mg/m$^2$ D1-5 Q3W) or capecitabine (1000 mg/ m$^2$ 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<br>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<br><br>Cohort A: Mismatch repair (MMR)-deficient colorectal cancers (CRC)<br>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<br>MMR-proficient CRC: ORR 0%, PFS 11%; median PFS 2.2 mo, OS 5.0 mo<br>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<br>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.

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MINIREVIEWS

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

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## 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.

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## 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)/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.

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ORIGINAL ARTICLE

Basic Study

# Intra-individual comparison of therapeutic responses to vascular disrupting agent CA4P between rodent primary and secondary liver cancers

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## 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.

Liu YW, De Keyper F, Feng YB, Chen F, Song SL, Swinnen J, Bormans G, Oyen R, Huang G, Ni YC. Intra-individual comparison of therapeutic responses to vascular disrupting agent CA4P between rodent primary and secondary liver cancers. *World J Gastroenterol* 2018; 24(25): 2710-2721 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i25/2710.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i25.2710

## 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 9$^{th}$ week until the largest liver tumor diameter reached more than 5 mm. A R1-tumor tissue block of 1 mm$^3$ 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 mm$^2$; 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 mm$^2$; 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 mm$^2$; 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

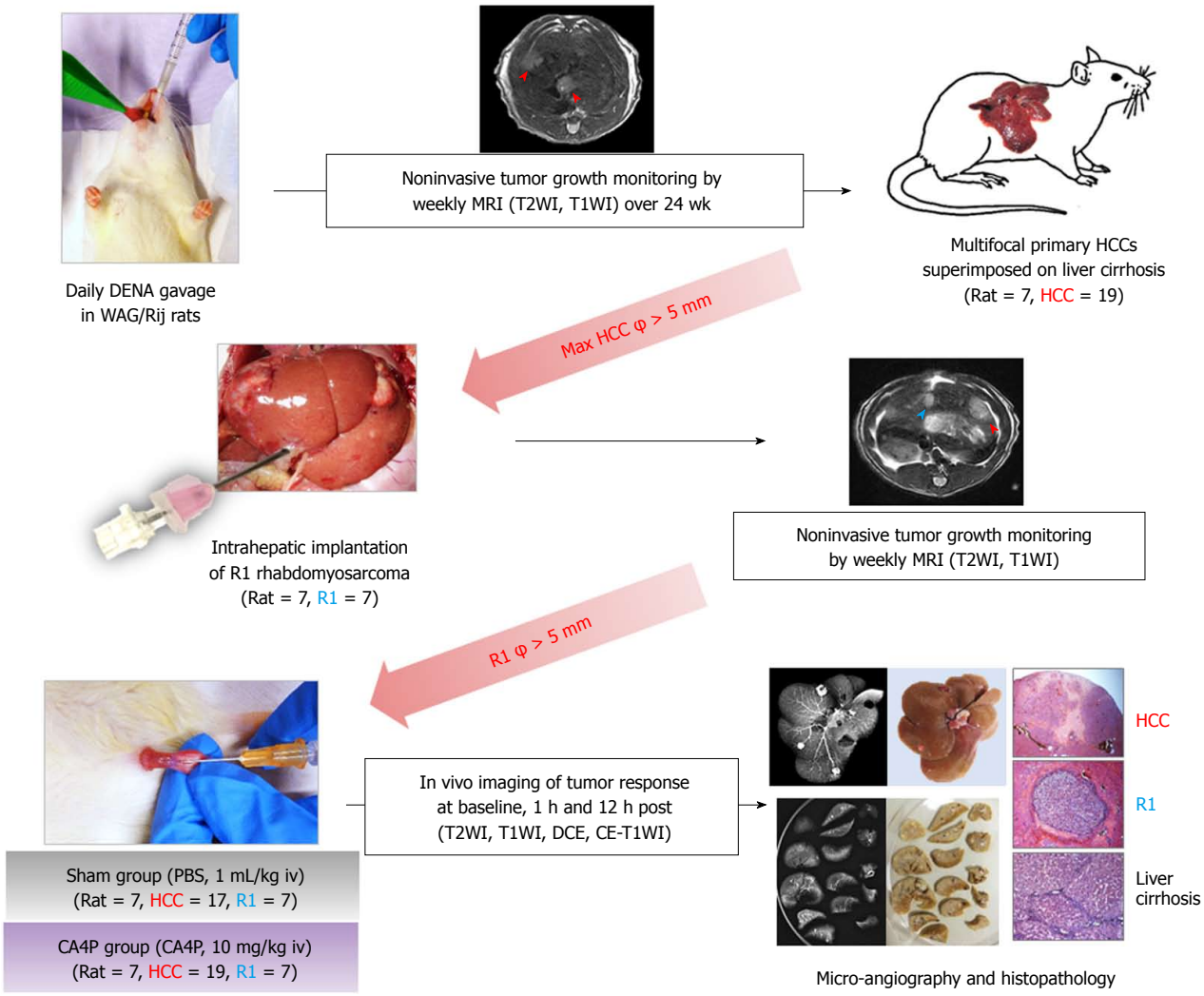


**Figure 1 Flow chart of experimental protocol.** φ: Diameter; CA4P: Combretastatin-A4-phosphate; CE: Contrast-enhanced; DCE: Dynamic contrast-enhanced; DENA: Diethylnitrosamine; HCC: Hepatocellular carcinoma; iv: Intravenous(ly); MRI: Magnetic resonance imaging; PBS: Phosphate-buffered saline; R1: R1 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 (%) = $\Sigma$ [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 allographs 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 allographs 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 allographs (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

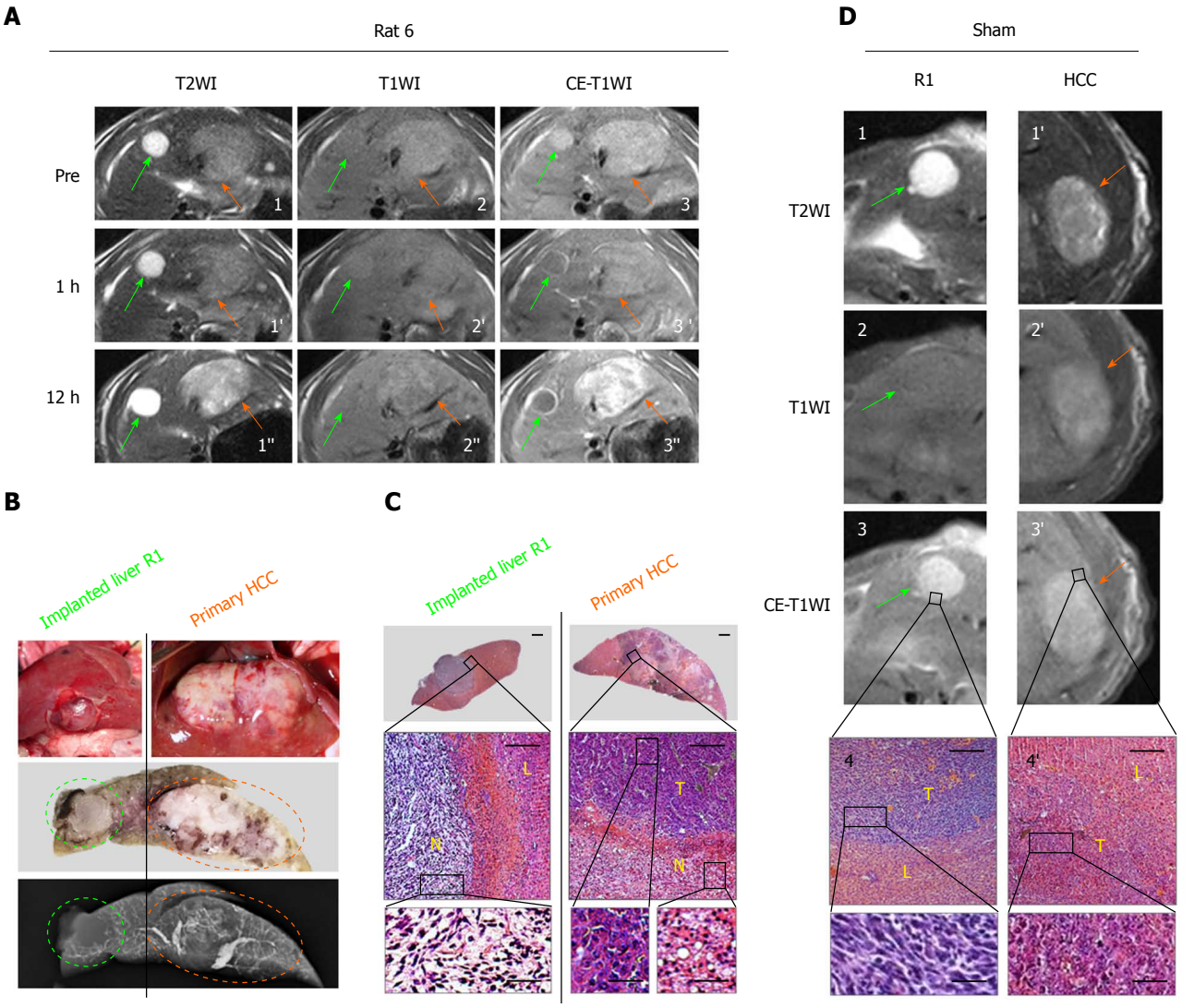


**Figure 2 Intra-individual comparison of therapeutic responses to combretastatin-A4-phosphate between a primary hepatocellular carcinoma and a hepatic R1 allograft located in different liver lobes.** A: T2WIs (1-1"), T1WIs (2-2') and CE-T1WIs (3-3") of an implanted R1-tumor (green arrows) and a primary 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

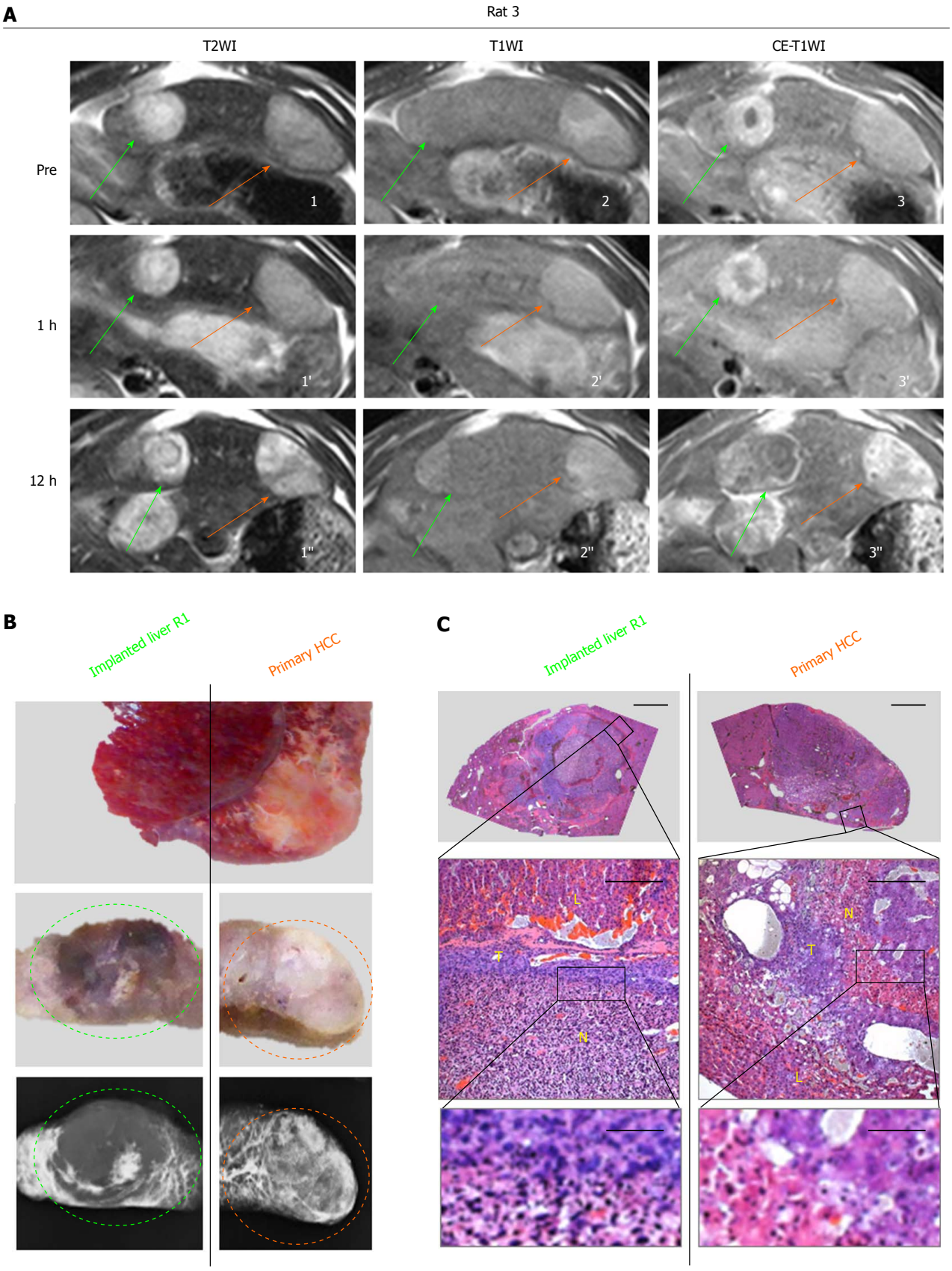


**Figure 3 Intra-individual comparison of therapeutic responses to combretastatin-A4-phosphate between a primary hepatocellular carcinoma and a hepatic R1 allograft distributed in the same liver lobe.** A: T2WIs (1-1"), T1WIs (2-2") and CE-T1WIs (3-3") of an implanted R1-tumor (green arrows) and a primary 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.

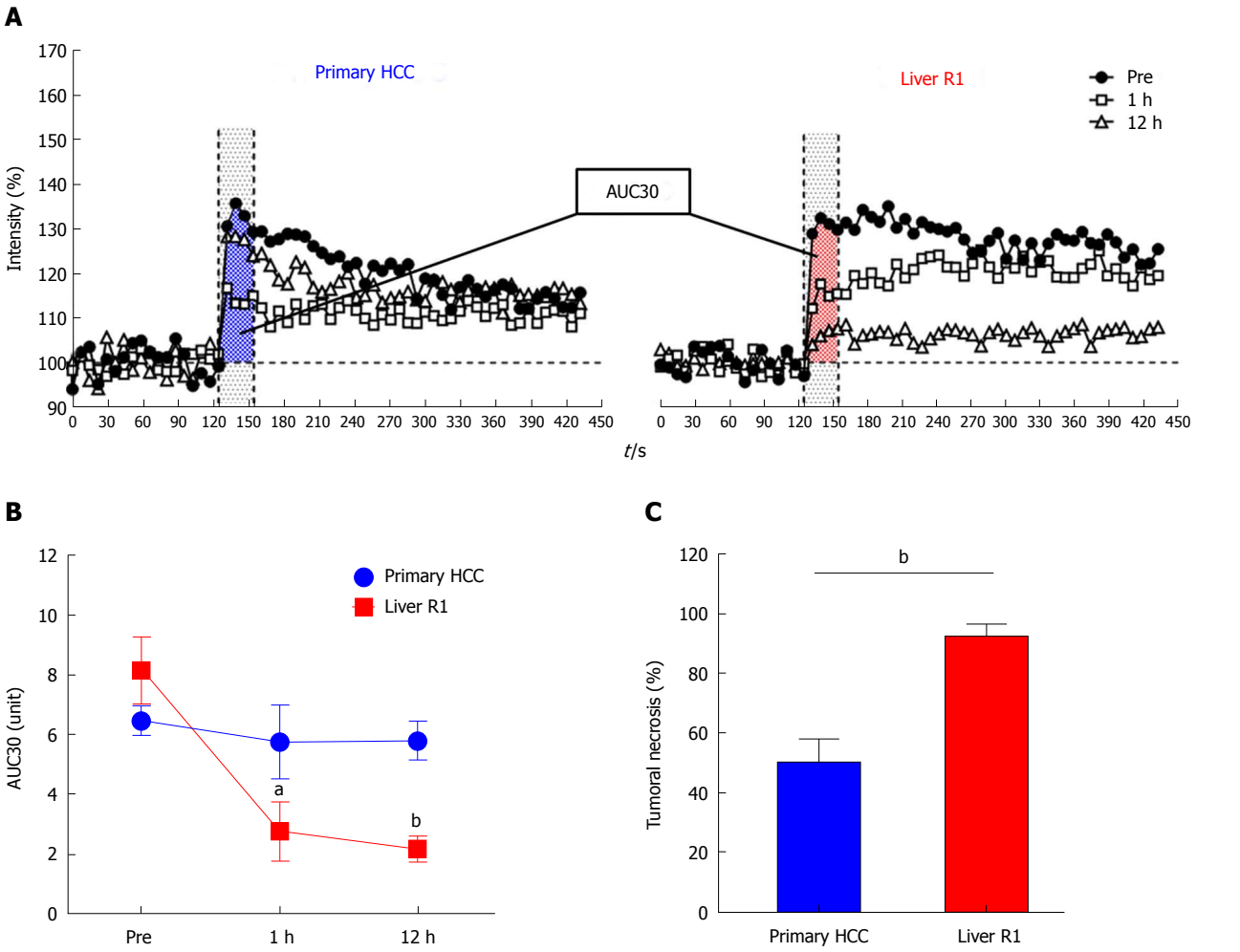


**Figure 4 Changes of semiquantitative dynamic contrast-enhanced parameter of primary hepatocellular carcinomas and implanted liver R1-tumors and quantification of combretastatin-A4-phosphate-induced tumoral necrosis.** A: Representative contrast enhancement-time curves of a primary HCC and a secondary 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.

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ORIGINAL ARTICLE

Retrospective Study

# Gastric cancer in Alaska Native people: A cancer health disparity

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## 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.

**Key words:** Alaska Native; Gastric cancer; *Helicobacter pylori*; Gender; Health disparities



**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.

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## 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) 7$^{th}$ 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,

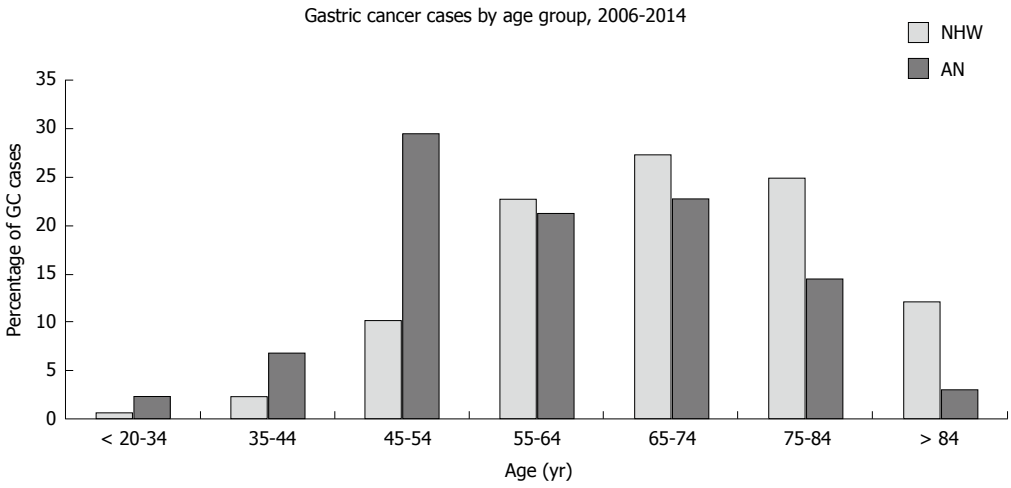


**Figure 1 Age group distribution for non-Hispanic White and Alaska Native patients diagnosed with gastric cancer 2006-2014.** AN: Alaska Native; NHW: Non-Hispanic White.

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.

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ORIGINAL ARTICLE

Observational Study

# Transforming growth factor-β and peripheral regulatory cells are negatively correlated with the overall survival of hepatocellular carcinoma

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## 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.

An Y, Gao S, Zhao WC, Qiu BA, Xia NX, Zhang PJ, Fan ZP. Transforming growth factor-β and peripheral regulatory cells are negatively correlated with the overall survival of hepatocellular carcinoma. *World J Gastroenterol* 2018; 24(25): 2733-2740 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i25/2733.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i25.2733

## 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 $2 \times 10^6$/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

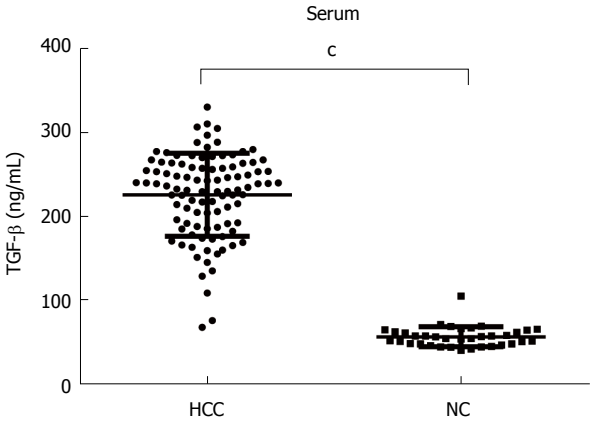


**Figure 1 Levels of transforming growth factor-β in the serum of hepatocellular carcinoma patients and normal controls.** °*P* < 0.001. TGF-β: Transforming growth factor-β; HCC: Hepatocellular carcinoma.

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

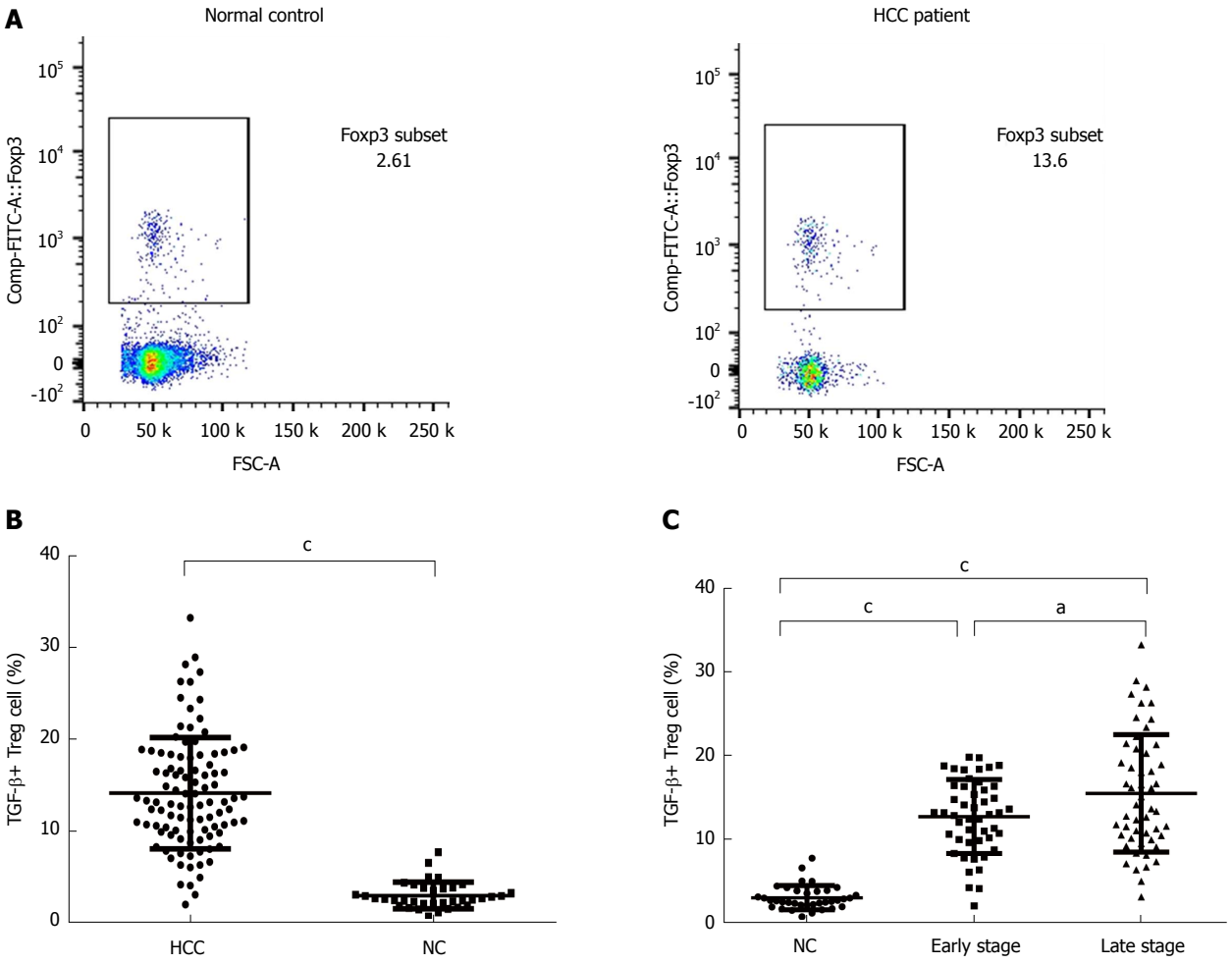


**Figure 2 Increase of transforming growth factor-β+ regulatory cells in the serum of hepatocellular carcinoma patients.** A: Representative plots for identification of the TGF-β+ Treg cells producing cells in PBMC; B: Analysis of the frequency of TGF-β+ Treg cell from the PBMCs of healthy donors in 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.

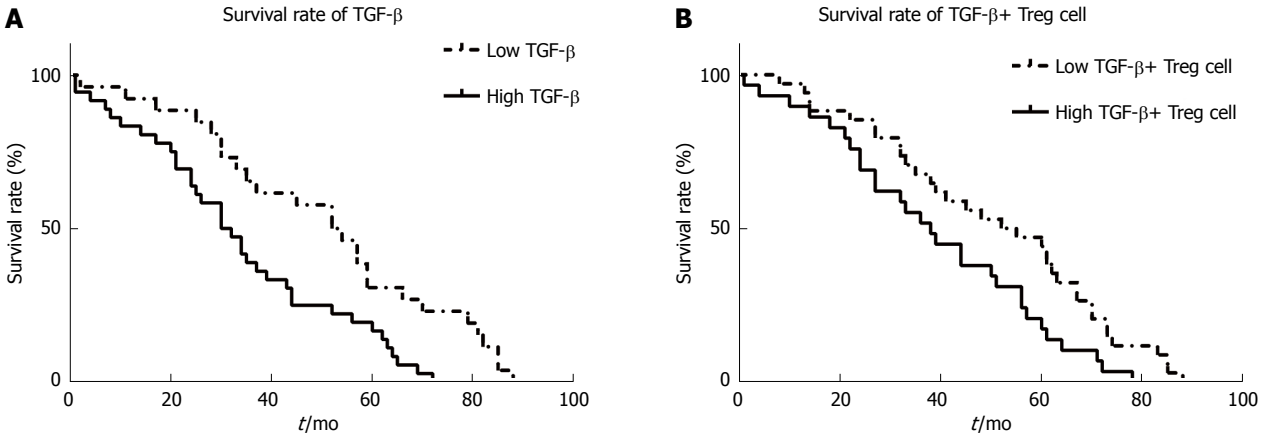


**Figure 3 Level of transforming growth factor-β and transforming growth factor-β+ regulatory cells affect 5-year survival for hepatocellular carcinoma patients.** The 5-year survival rates for HCC patients with a high and low level of TGF-β (A), and TGF-β+ Treg cells (B) in their PBMCs. The survival 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

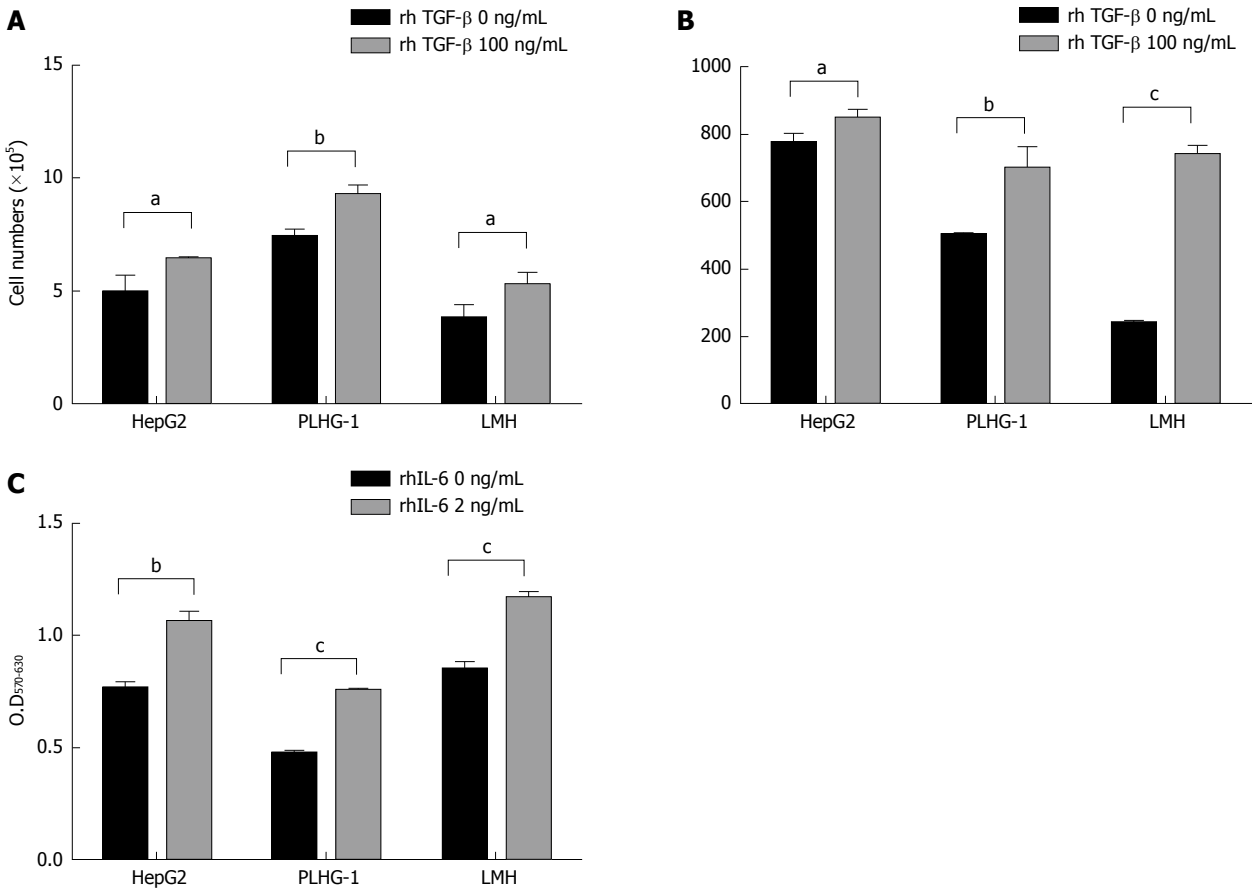


**Figure 4 Transforming growth factor-β can promote the proliferation of tumor cells and increase the level of interleukin-6 in hepatocellular carcinoma cell lines production.** A: The cell numbers of the three HCC cell lines after treatment with 100 ng/mL rhTGF-β for 48 h; B: The three HCC culture supernatants 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]. Thl7 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.

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SYSTEMATIC REVIEWS

# Current global trends in the incidence of pediatric-onset inflammatory bowel disease

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## 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.

Sýkora J, Pomahačová R, Kreslová M, Cvalínová D, Štych P, Schwarz J. Current global trends in the incidence of pediatric-onset inflammatory bowel disease. *World J Gastroenterol* 2018; 24(25): 2741-2763 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i25/2741.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i25.2741

## 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.

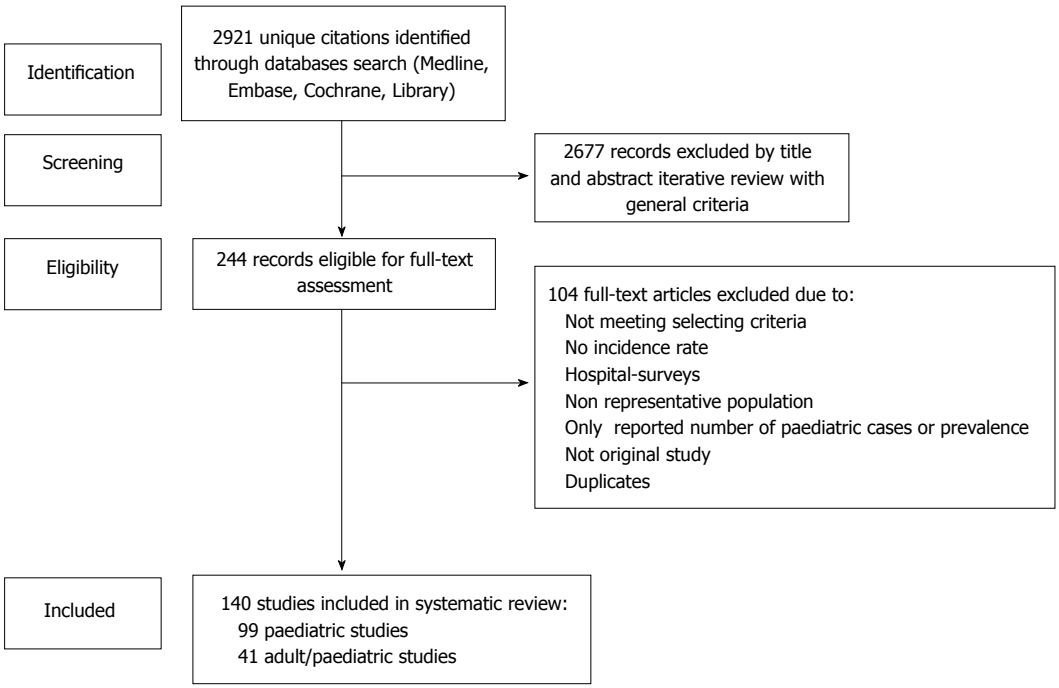


**Figure 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses flowchart diagram for study selection.**

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 | $9.0^{2}$a | $2.5^{2}$a | $5^{2}$a | $1.5^{2}$a | 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 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 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 | | 14-11a$^2$c | 4-6a$^2$c | 0-1.5a$^2$c | 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 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 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 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 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 | < 15 | 2002-2003 | 2.9d | 1.9d | 0.5d | | P | 2008 | Yap *et al*[26] | A |
| Sousthern Europe | | | | | | | | | | |
| Italy/Florence | < 15 | 1978-1992 | | 1.9-3.4a | 3.8-9.6a | | R | 1996 | Trallori *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 ($P < 0.05$)

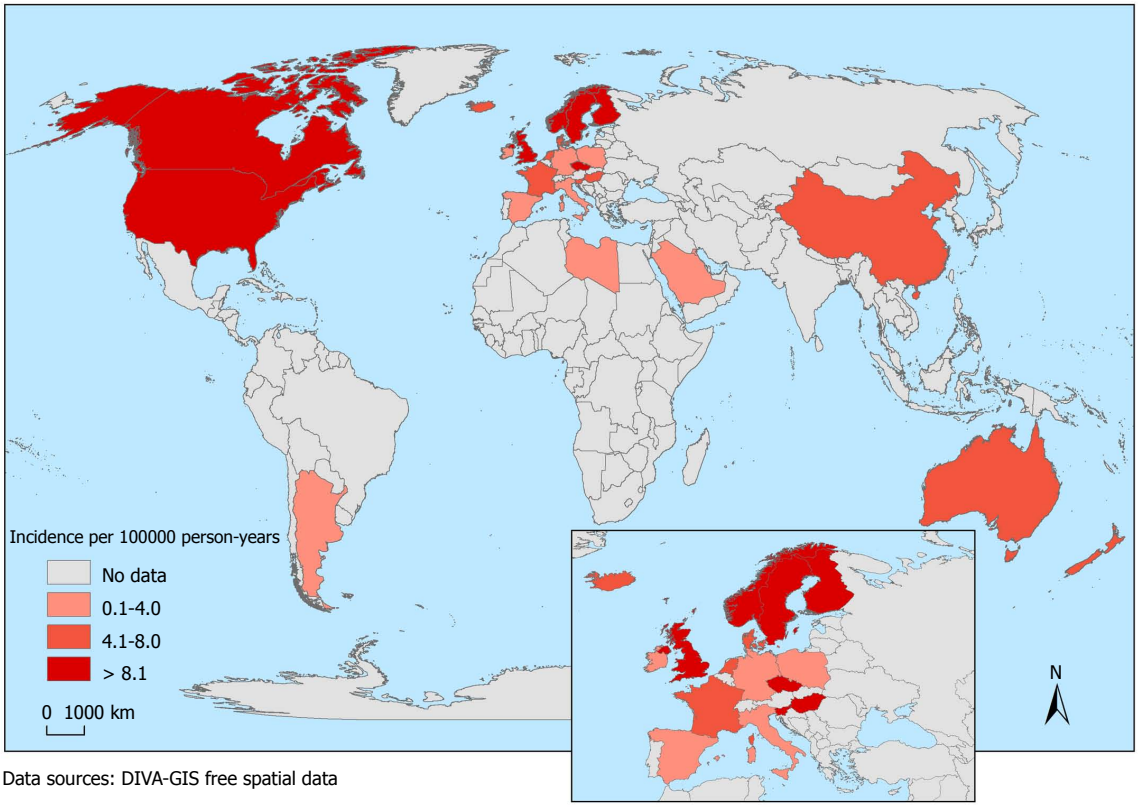


**Figure 2 Worldwide pediatric inflammatory bowel disease incidence rates.** Inflammatory bowel disease (IBD) choropleth map of global incidence of pediatric IBD divided into four colors representing unknown, low, indeterminate and high occurrence of disease. Grey reflects absence of data.

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

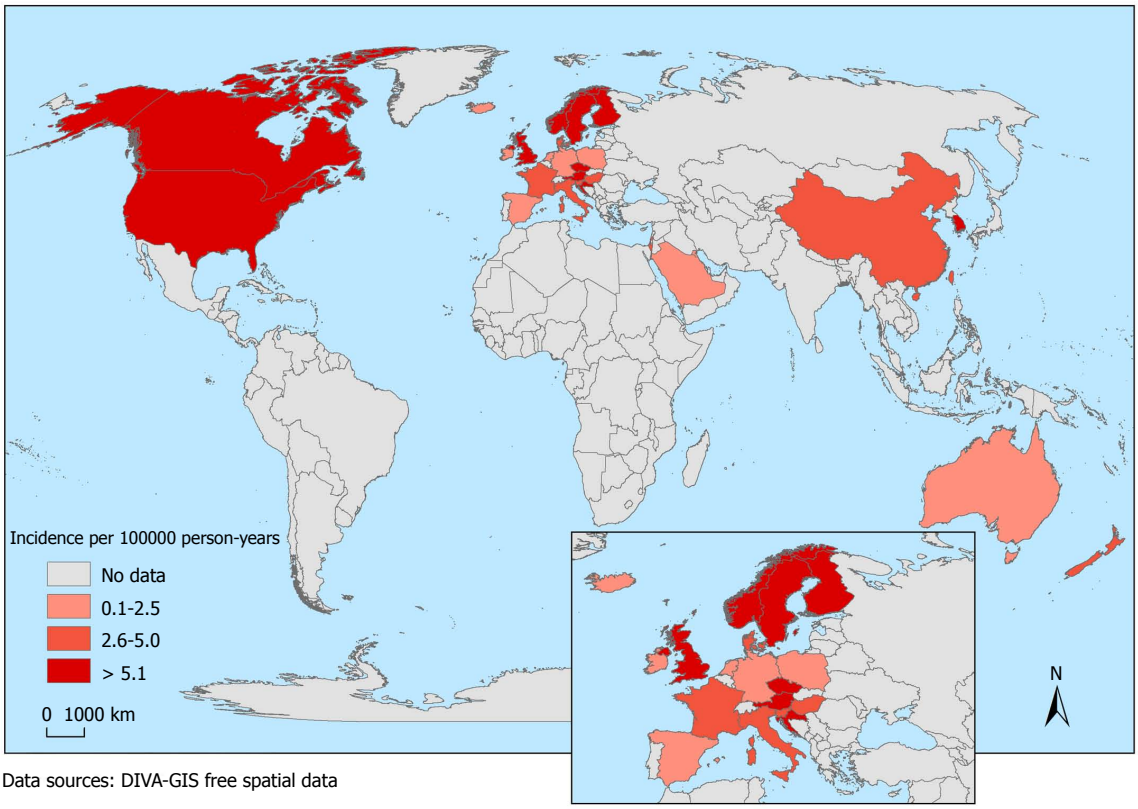


**Figure 3 Worldwide pediatric Crohn's disease incidence rate.** Crohn's disease choropleth map of global incidence of pediatric inflammatory bowel disease divided into four colors representing unknown, low, indeterminate and high occurrence of disease. Grey reflects absence of data.

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], highlighting 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

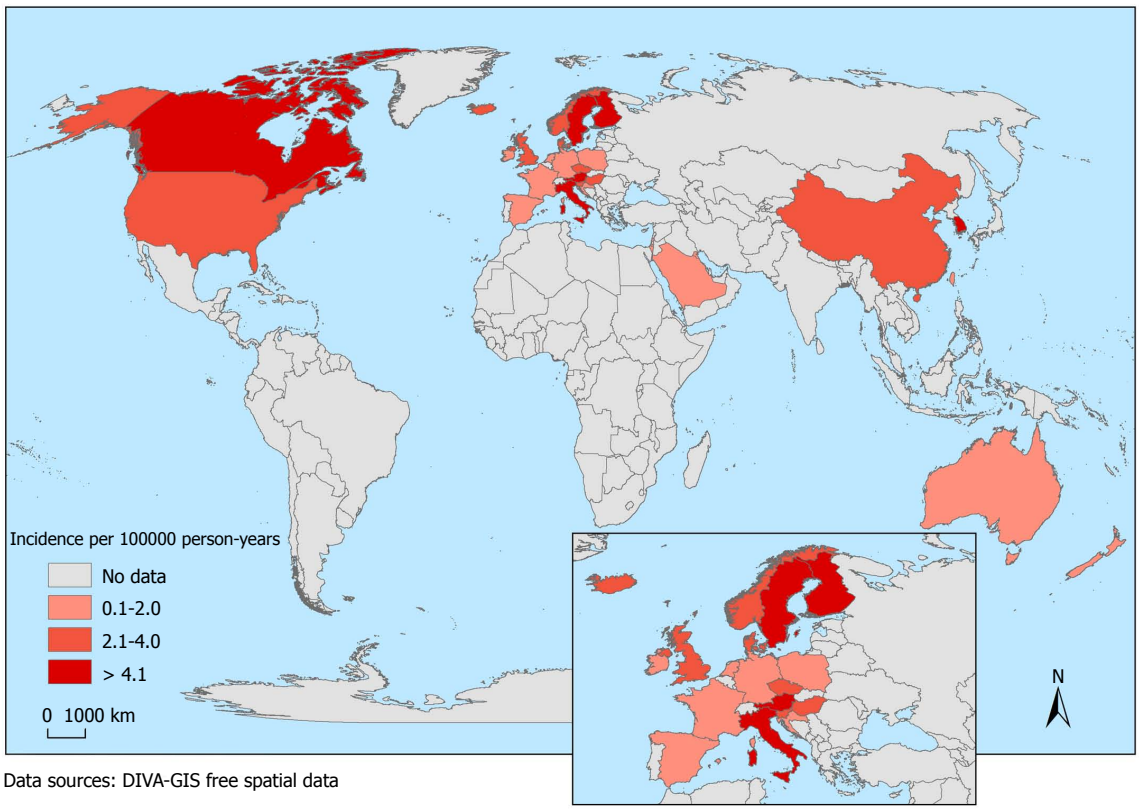


**Figure 4 Worldwide pediatric ulcerative colitis incidence rate.** Ulcerative colitis choropleth map of global incidence of pediatric inflammatory bowel disease divided into four colors representing unknown, low, indeterminate and high occurrence of disease. Grey reflects absence of data.

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

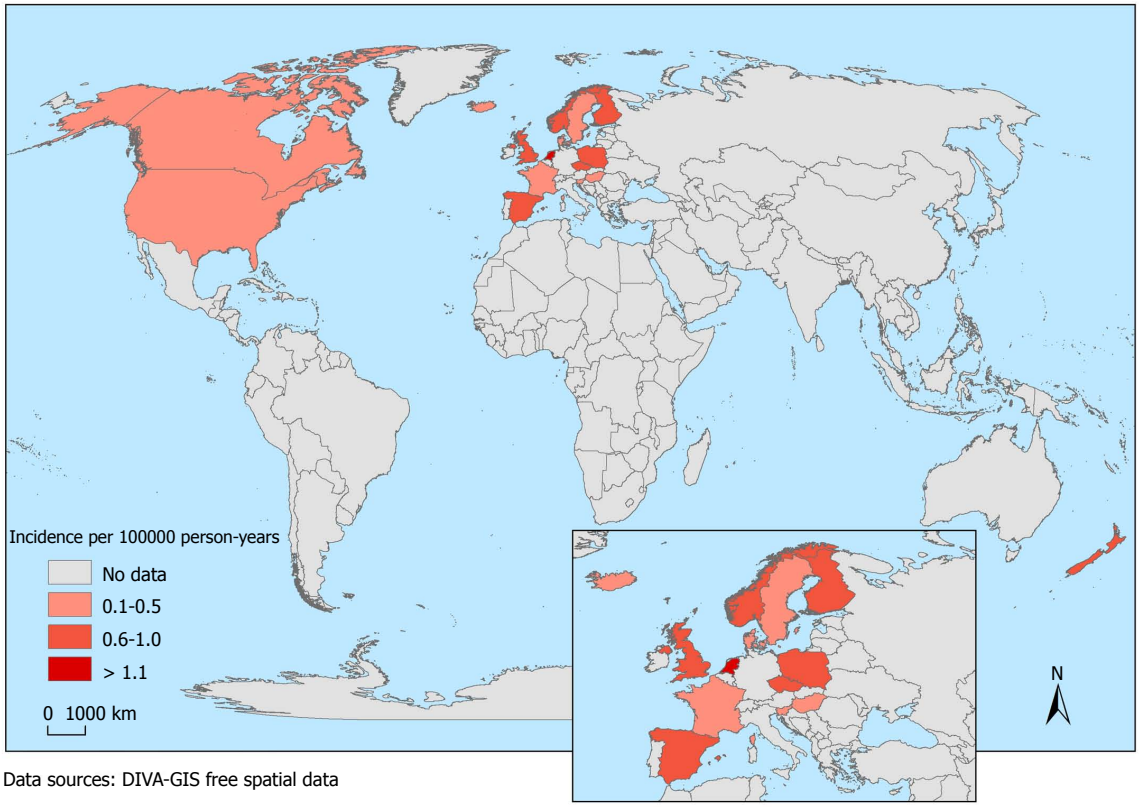


**Figure 5 Worldwide pediatric inflammatory bowel disease-unclassified incidence rate.** Inflammatory bowel disease-unclassified choropleth map of global incidence of pediatric inflammatory bowel disease divided into four colors representing unknown, low, indeterminate and high occurrence of disease. 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.

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*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)

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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.

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## 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.

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## 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:

$$\widehat{ASD} = \arcsin\sqrt{\frac{a}{N_T}} - \arcsin\sqrt{\frac{b}{N_C}} \quad \text{(Equation 1)}$$

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:

$$\text{Log}\widehat{OR}_{Peto} = (a - \frac{a+b}{n_T+n_C} * n_T)^* \frac{(n_T+n_C)^2/(n_T+n_C-1)}{(a+b)(n_T-a+n_C-b)n_Tn_C} \quad \text{(Equation 2)}$$

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.

$$\text{EBC} = n \times \textit{Follow up duration (years)} \times \frac{20 \text{ cases}}{100{,}000 \text{ person-years}} \quad \text{(Equation 3)}$$

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*.

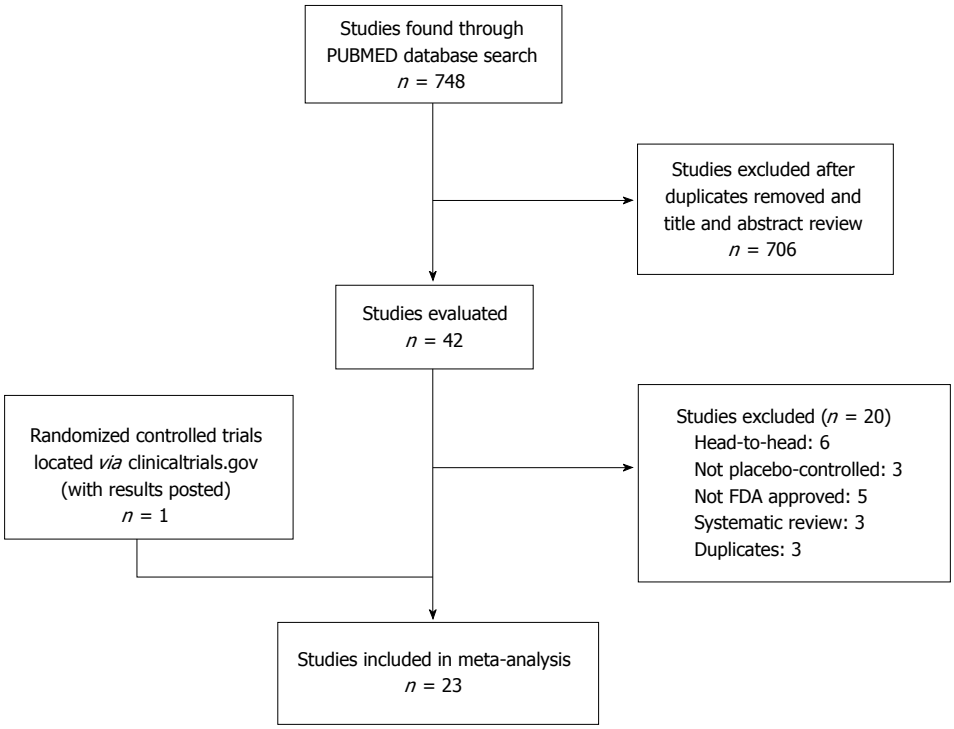


**Figure 1 Evidence collection and selection.**

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

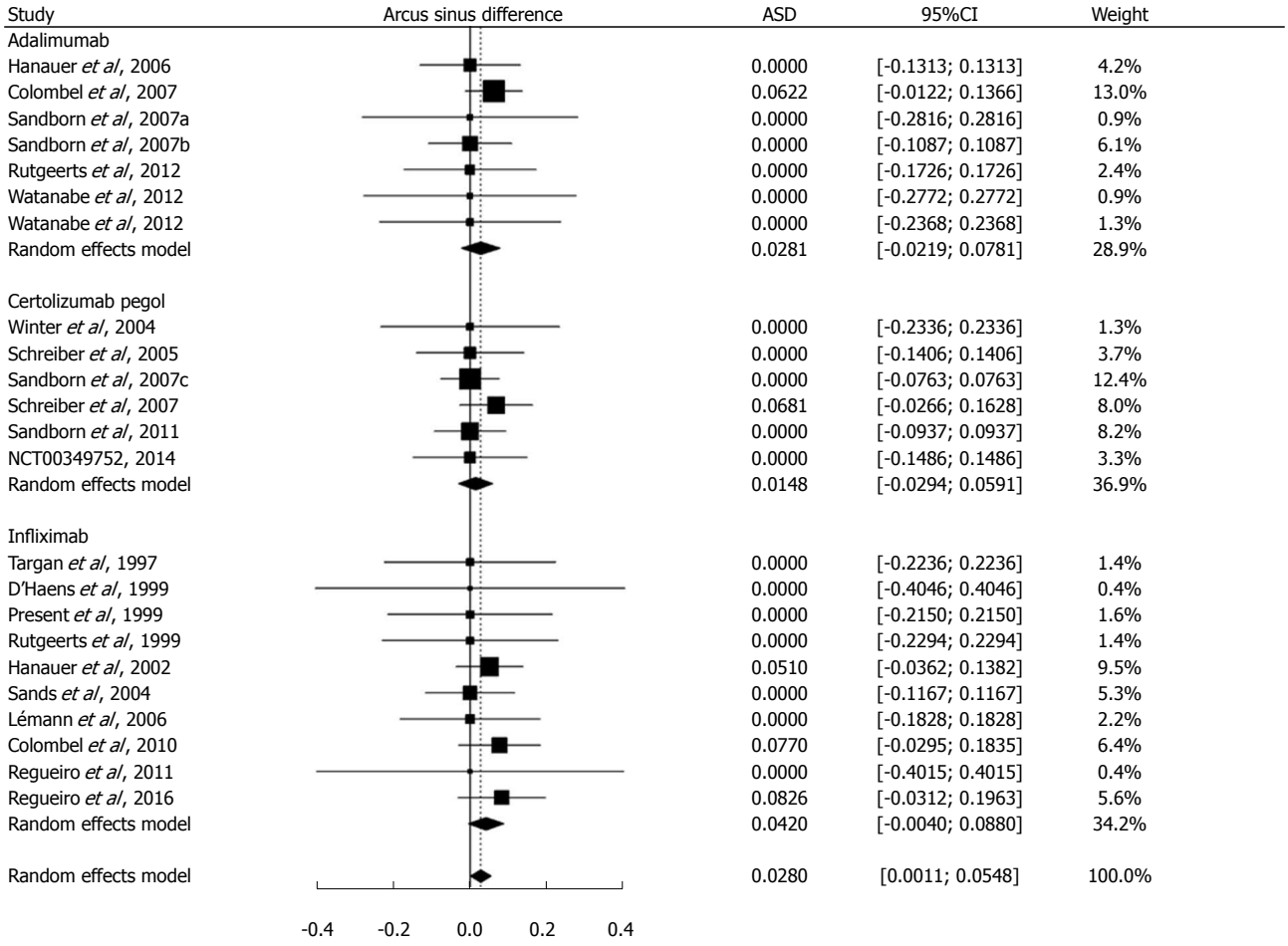


**Figure 2 Difference of the risk of *Mycobacterium tuberculosis* infection between patients with Crohn's disease treated with tumor necrosis factor-alpha inhibitors and those treated with placebo.** Risk difference was calculated after arcsine transformation of *Mycobacterium tuberculosis* incidence (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.

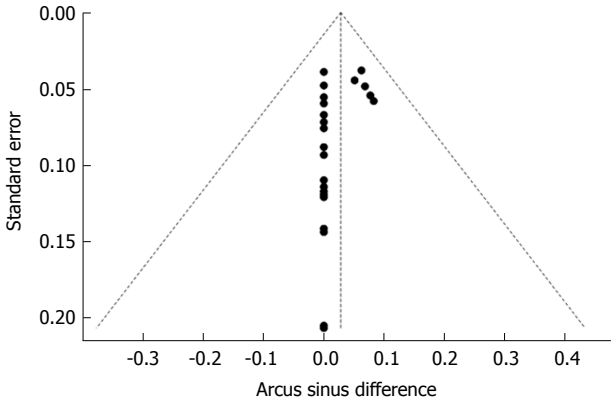


**Figure 3 Relationship between the estimated *Mycobacterium tuberculosis* infection risk difference and the corresponding standard error of the estimate.** Risk difference was calculated after arcsine transformation of *Mycobacterium tuberculosis* incidence (Arcus sinus difference, ASD). The dashed 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

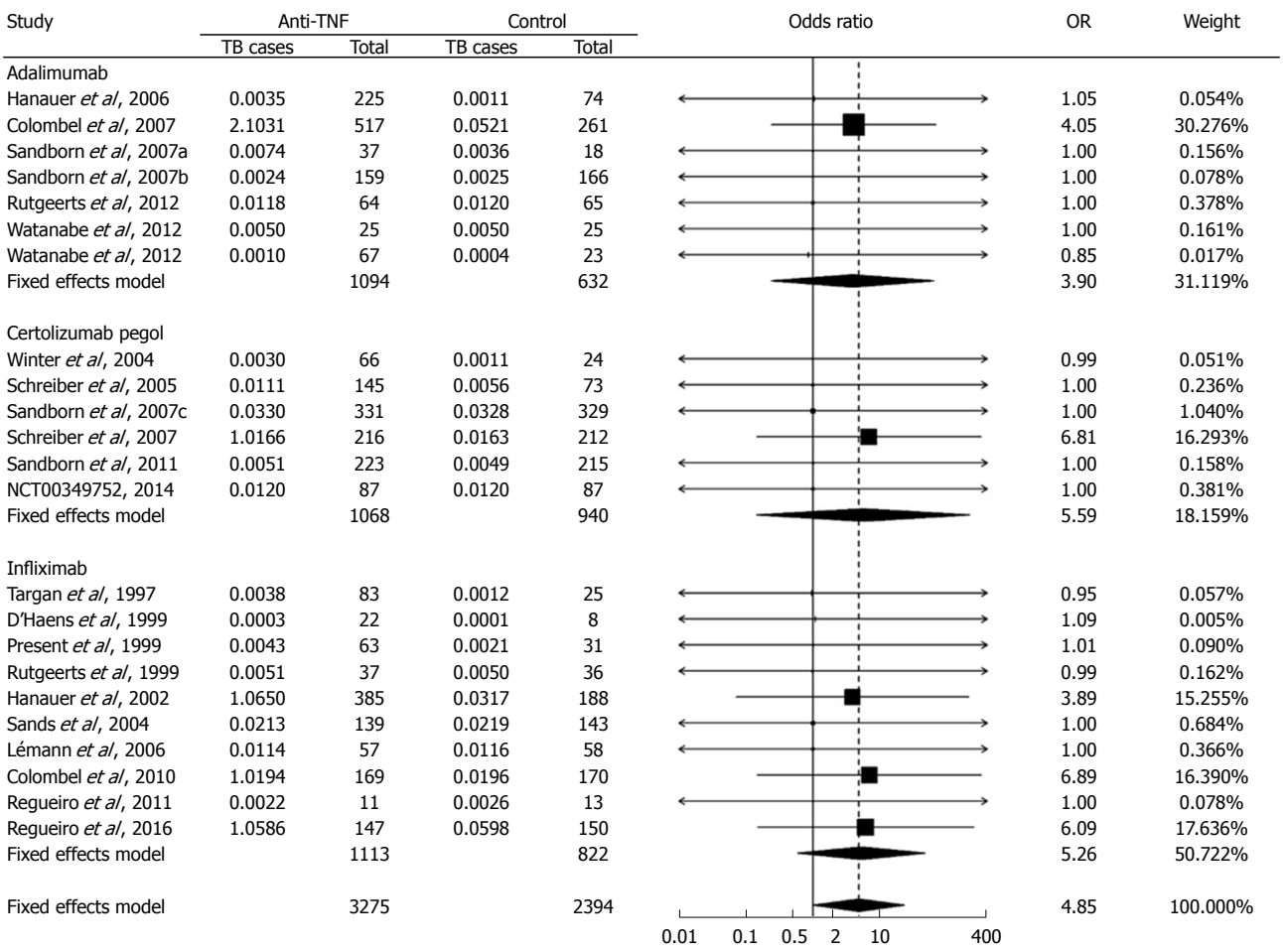


**Figure 4 Odds of patients developing active *Mycobacterium tuberculosis* when treated with tumor necrosis factor-alpha inhibitors for Crohn's disease relative to those treated with placebo.** Odds ratio (OR) was calculated using the Yusuf-Peto method and indicated by the numbers on the x-axis. Number 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.

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CASE REPORT

# Liposarcoma of the stomach: Report of two cases and review of the literature

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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.

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## 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.

Kang WZ, Xue LY, Wang GQ, Ma FH, Feng XL, Guo L, Li Y, Li WK, Tian YT. Liposarcoma of the stomach: Report of two cases and review of the literature. *World J Gastroenterol* 2018; 24(25): 2776-2784 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i25/2776.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i25.2776

## 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.

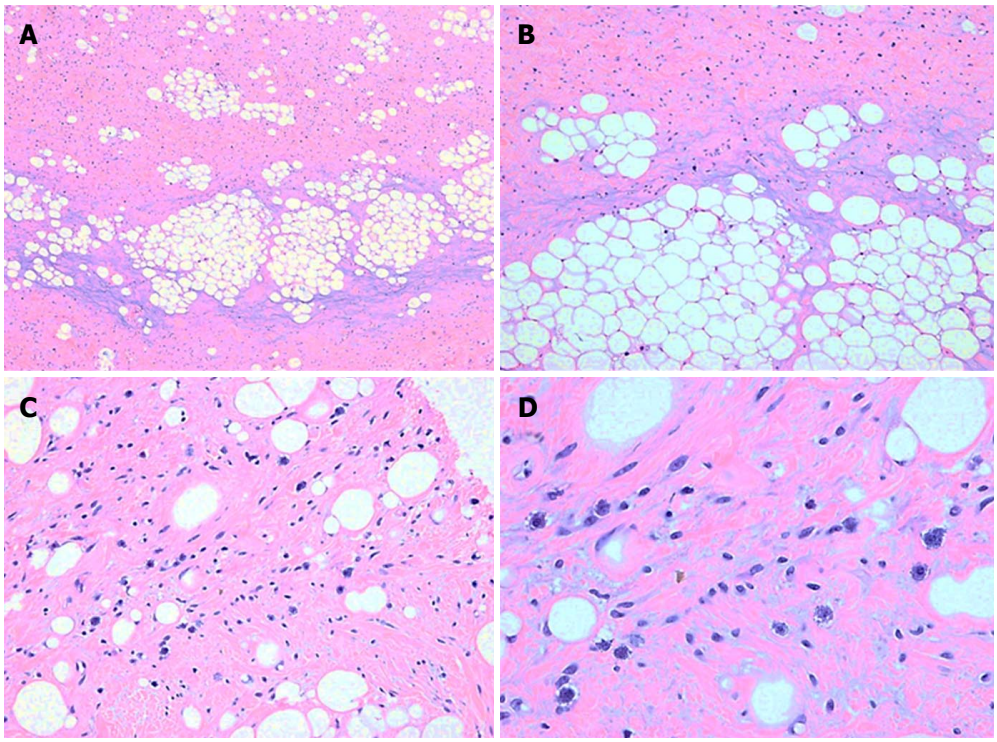


**Figure 1 Histological finding.** Low magnification shows that the immature fat cells are interspersed among smooth muscle tissues (A: HE, × 40 and B: HE; × 100). The large nuclear dark-stained lipoblast, which appears as a mononuclear or multinucleated cell with one or more cytoplasmic vacuoles, are seen under high magnification (C; HE; × 200 and D: HE; × 400).

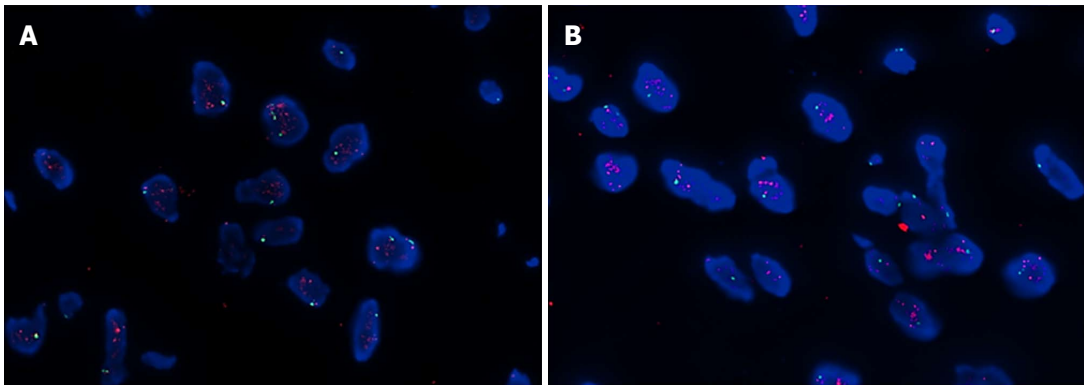


**Figure 2 Fluorescence *in situ* hybridization detection shows amplification of *MDM2* gene (A and B), case 1.**

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

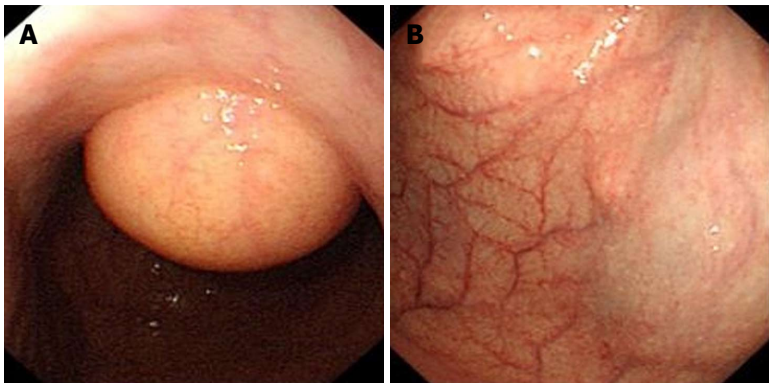


**Figure 3 A limited knurl was distributed from lower part of the gastric body to the corner of the stomach (A), and a knurl was also found in the gastric fundus (B).**

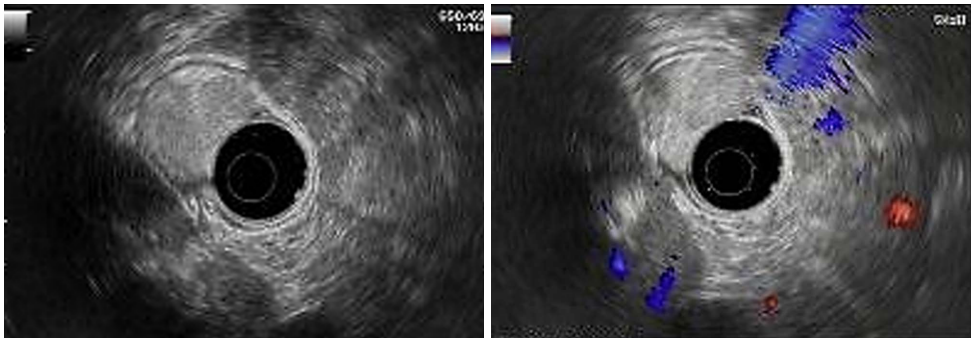

**Figure 4 Endoscopic ultrasound examination located the tumor mainly in the submucosa of the gastric wall.**

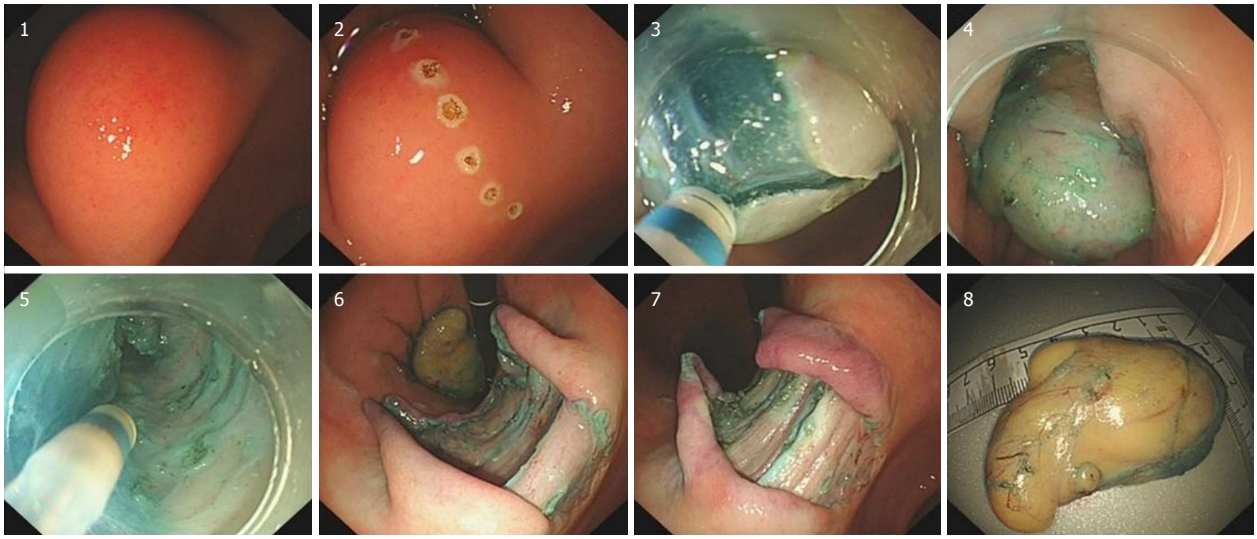


**Figure 5 Process of the endoscopic submucosal dissection.**

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].

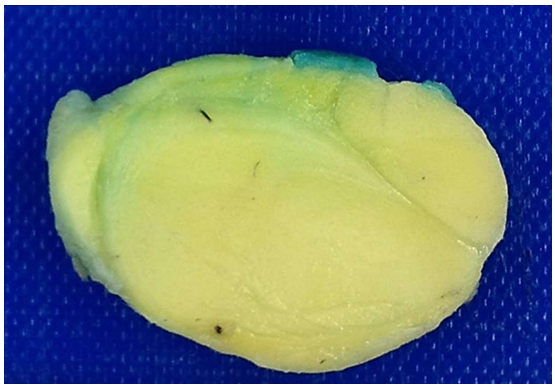

**Figure 6 Gross specimen of the tumor.**

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

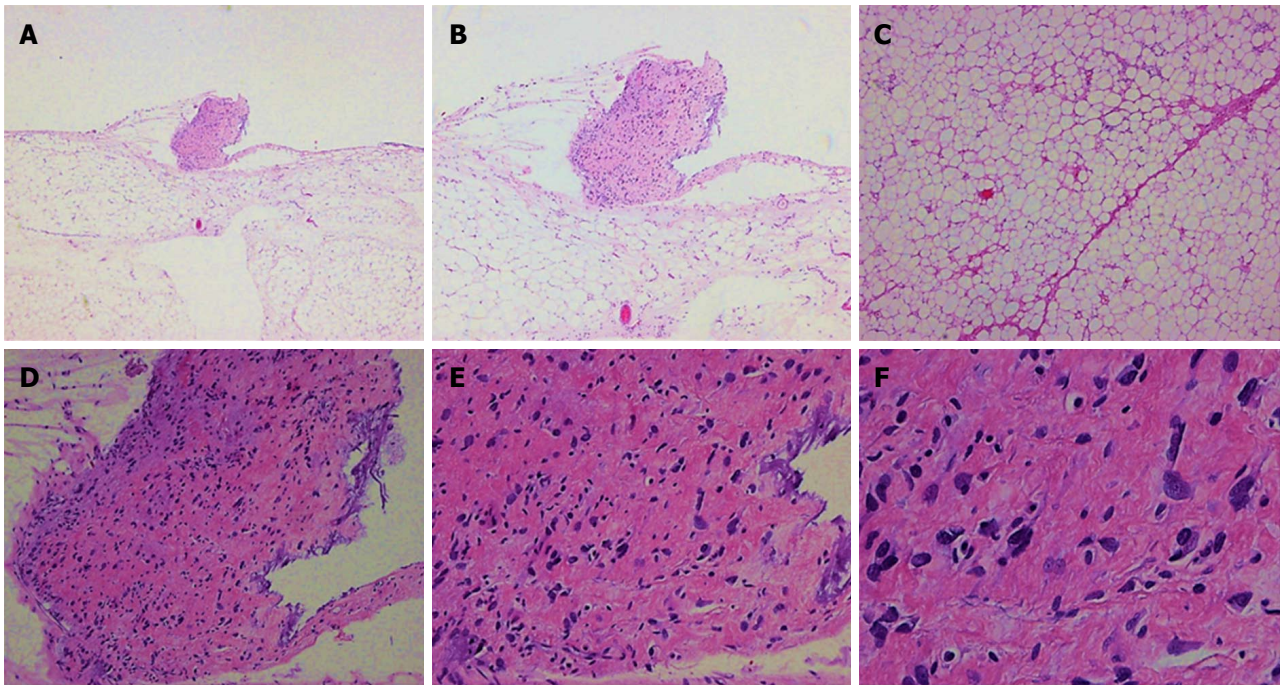


**Figure 7 Histological findings.** Under low magnification, irregular cell cluster nests were seen around the mature adipocytes (A: HE; × 20 and B: HE; × 40); intermediate magnification and high magnification showed that the heteromorphic cell nests consisted of large nuclear dark-stained tumor cells, with distinct cell shapes, irregular cell morphologies, and visible Mitosis icon (C: HE; × 100, D: HE; × 100, E: HE; × 200, and F: HE; × 400).

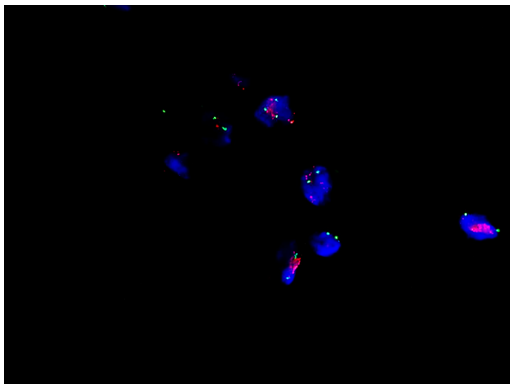

**Figure 8 Fluorescence in situ hybridization detection shows *MDM2* gene amplification, case 2.**

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]. Pleiomorphic 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.

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